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INDEX

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CONTENTS OF VOLUME 52, No. 1, 1952

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PAGE

Nomenclature of amino-acids and vitamins 1

The relation between pyridoxin and tryptophan metabolism, studied in the rat. By C. E. Dalgliesh. (With four figures) 3

The virulence-enhancing factor of mucins. 5. The different components of the 'third factor' involved in virulence enhancement. By H. Smith, R. C. Gallop and J. L. Stanley. (With one figure) . . 15

Addendum—Statistical treatment of data regarding the synergism of mixtures of polysaccharides A and C. By S. Peto 22

The virulence-enhancing factor of mucins. 6. Identification of a heparin as the main component of the third factor involved in virulence enhancement. By H. Smith, R. C. Gallop, P. W. Harris-Smith and J. L. Stanley. (With three figures) 23

Addendum—Physico-chemical examination of polysaccharide A. By J. M. Creeth and B. R. Record. (With two figures) 30

The oxidation of nicotinic acid by *Pseudomonas fluorescens*. By A. Pinsky and M. Michaelis. (With one figure) 33

o-Phenylenediamine and 1:2-diamino-4-nitrobenzene as reagents for α-keto acids. By D. J. D. Hockenhull and G. D. Floodgate 38

The metabolism of succinic acid in the rumen of the sheep. By A. Kaars Sijpesteijn and S. R. Elsden. (With two figures) 41

Studies on cholinesterase. 8. Determination of reaction velocity constants with a reversible inhibitor of pseudo-cholinesterase. By D. K. Myers. (With five figures) 46

Volatile acids in the secretion products (hair grease) of the skin. By E. Brouwer and H. J. Nijkamp . . 54

The occurrence of growth factors for *Lactobacillus leichmannii*, *Streptococcus faecalis* and *Leuconostoc citrovorum* in the tissues of pernicious anaemia patients and controls. By R. H. Girdwood . . . 58

Some observations on the hydrolysis and extraction of formaldehydogenic corticosteroids. By R. I. S. Bayliss 63

The content of glycogenolytic factor in some insulin samples. By G. Audy and M. Kerly. (With one figure) 70

Appendix—Statistical examination of multiple-dose assays. By N. W. Please 75

The content of glycogenolytic factor in pancreas from different species. By G. Audy and M. Kerly. (With one figure) 77

The effect of varying amounts of dietary cholesterol and of choline upon liver lipids. By J. H. Ridout, C. C. Lucas, J. M. Patterson and C. H. Best. (With two figures) 79

The analysis of hair keratin. 1. Application of microbiological techniques to hydrolysates of human hair. By J. M. Lang and C. C. Lucas 84

Further isotopic studies on haemoglobin formation in the rat and rabbit. By H. M. Muir, A. Neuberger and J. C. Perrone 87

Lignin in young plants. By H. Meyer and A. Bondi 95

The estimation of serum bilirubin. By J. Patterson, J. Swale and C. Maggs. (With three figures) . 100

Lipovitellin. 2. The influence of water on the stability of lipovitellin and the effects of freezing and of drying. By C. H. Lea and J. C. Hawke. (With ten figures) 105

The effects and the fate of malononitrile and related compounds in animal tissues. By J. Stern, H. Weil-Malherbe and R. H. Green. (With four figures) 114

The oxidation of manganese by enzyme systems. By R. H. Kenten and P. J. G. Mann. (With two figures) 125

Hydrogen peroxide formation in oxidations catalysed by plant α-hydroxyacid oxidase. By R. H. Kenten and P. J. G. Mann. (With two figures) 130

The biochemistry of the nitrifying organisms. 1. The ammonia-oxidizing systems of *Nitrosomonas*. By H. Lees. (With four figures) 134

The biochemistry of the nitrifying organisms. 2. The free-energy efficiency of *Nitrosomonas*. By T. Hofman and H. Lees. (With one figure) 140

The ultraviolet absorption spectrum of ribonuclease. By D. Shugar. (With five figures) 142

Further observations on the preparation and composition of the hyaluronic acid complex of ox synovial fluid. By A. G. Ogston and J. E. Stanier. (With three figures) 149

Separation of adenosine phosphates by paper chromatography and the equilibrium constant of the myokinase system. By L. V. Eggleston and R. Hems. (With one figure) 156

Proceedings of the Biochemical Society

PAGE

The dialuric acid haemolysis test for vitamin E deficiency in rats. By D. H. Heard, T. Moore and I. M. Sharman i

The effects of deoxyribonucleic acid and suramin on β-glucuronidase activity. By E. E. B. Smith and G. T. Mills i

The partition of carotenoids and vitamin A in the milk of cows and goats throughout lactation. By R. Chanda ii

The amino-acid sequence in the glycyl chain of insulin. By F. Sanger and E. O. P. Thompson . . iii

Further observations on the amide and free carboxyl groups of insulin. By A. C. Chibnall and M. W. Rees iii

Determination of the amino-acid sequence in peptides. By J. L. Bailey iv

7-Dehydrocholesterol in the sexual organs of the rat. By T. Moore and R. J. Ward v

The reaction of haemoglobin with hydrogen peroxide. By K. Dalziel and J. R. P. O'Brien . . . v

The reaction of methaemoglobin with hydrogen peroxide. By K. Dalziel and J. R. P. O'Brien . . vi

The metabolism of anthranilic acid in the rabbit and rat. By F. Charconnet-Harding, C. E. Dalgliesh and A. Neuberger vii

Riboflavin and tryptophan metabolism in the rat. By F. Charconnet-Harding, C. E. Dalgliesh and A. Neuberger vii

Some properties of vitamin B_{12}-like factors from calf faeces. 3. Further biological properties and inter-relationships. By J. E. Ford, S. K. Kon and J. W. G. Porter viii

The metabolism of *n*-valeric acid and some branched chain acids by sheep tissues *in vitro*. By E. F. Annison and R. J. Pennington ix

Attempts to render growth hormone non-diabetogenic. By E. Reid ix

The direct determination of nitrogen in protein fractions separated by electrophoresis in filter paper. By A. B. Anderson x

Enzymic oxidation of 5-hydroxytryptamine in mammalian and cephalopod tissue. By H. Blaschko . x

The influence of growth hormone on the protein composition of rat muscle. By B. J. Hume . . . xi

Blood adrenaline levels during insulin hypoglycaemia. By H. Weil-Malherbe xi

Vitamin A and the resistance of rats to protein deficiency. By T. Moore, I. M. Sharman and R. J. Ward xii

Precursors of porphyrin in the urine in idiopathic porphyria. By F. K. Herbert xii

The 'Chromatocoil'—a space-saving device for paper chromatograms. By V. Schwarz xiii

Cytochrome components in chloroplasts. By H. E. Davenport xiii

Glycosides and a glycosidase in the madder plant. By A. R. Trim and R. Hill xiii

INDEX OF AUTHORS

	PAGE
Anderson, A. B.	x
Annison, E. F.	ix
Audy, G.	70, 77
Bailey, J. L.	iv
Bayliss, R. I. S.	63
Best, C. H.	79
Blaschko, H.	x
Bondi, A.	95
Brouwer, E.	54
Chanda, R.	ii
Charconnet-Harding, F.	vii
Chibnall, A. C.	iii
Creeth, J. M.	30
Dalgliesh, C. E.	3, vii
Dalziel, K.	v, vi
Davenport, H. E.	xiii
Eggleston, L. V.	156
Elsden, S. R.	41
Floodgate, G. D.	38
Ford, J. E.	viii
Gallop, R. C.	15, 23
Girdwood, R. H.	58
Green, R. H.	114
Harris-Smith, P. W.	23
Hawke, J. C.	105
Heard, D. H.	i
Hems, R.	156
Herbert, F. K.	xii
Hill, R.	xiii
Hockenhull, D. J. D.	38
Hofman, T.	140
Hume, B. J.	xi
Kenten, R. H.	125, 130
Kerly, M.	70, 77
Kon, S. K.	viii
Lang, J. M.	84
Lea, C. H.	105
Lees, H.	134, 140
Lucas, C. C.	79, 84
Maggs, C.	100
Mann, P. J. G.	125, 130
Meyer, H.	95
Michaelis, M.	33
Mills, G. T.	i
Moore, T.	i, v, xii
Muir, H. M.	87
Myers, D. K.	46
Neuberger, A.	87, vii
Nijkamp, H. J.	54
O'Brien, J. R. P.	v, vi
Ogston, A. G.	149
Patterson, J.	100
Patterson, J. M.	79
Pennington, R. J.	ix
Perrone, J. C.	87
Peto, S.	22
Pinsky, A.	33
Please, N. W.	75
Porter, J. W. G.	viii
Record, B. R.	30
Rees, M. W.	iii
Reid, E.	ix
Ridout, J. H.	79
Sanger, F.	iii
Schwarz, V.	xiii
Sharman, I. M.	i, xii
Shugar, D.	142
Sijpesteijn, A. K.	41
Smith, E. E. B.	i
Smith, H.	15, 23
Stanier, J. E.	149
Stanley, J. L.	15, 23
Stern, J.	114
Swale, J.	100
Thompson, E. O. P.	iii
Trim, A. R.	xiii
Ward, R. J.	v, xii
Weil-Malherbe, H.	114, xi

Nomenclature of Amino-acids and Vitamins

The Commission for the Reform of Biochemical Nomenclature of the International Union of Pure and Applied Chemistry has adopted Rules 1–5, 8 and 9, previously agreed between British and American chemists and biochemists and published (1948) in the *Biochem. J.* **42**, 1

which have trivial names; *allo*hydroxy-L-proline, *allo*-hydroxy-D-proline, *allo*hydroxy-L-lysine, *allo*hydroxy-D-lysine, and similarly for the enantiomorphs of the diastereomers named as derivatives of amino-acids which have trivial names. Where the position of the substituent group is designated the names take the form *allo*-4-hydroxy-L-

Its enantiomorph is denoted by the prefix *allo* and the prefix D. Where the name is wholly trivial, the L or D is placed before the prefix *allo* which is, in turn, attached to the parent name; but in names which contain one or more prefixes denoting substitution, the *allo*- is placed before this prefix and the L- or D- is placed as stated in section (*a*).

Examples: L-*allo*threonine, D-*allo*threonine, L-*allo*isoleucine, D-*allo*isoleucine for the pairs of enantiomorphs of the diastereomers of the amino-acids of protein origin

Where the name contains one or more prefixes denoting substitution and where specification both of configuration and of position of substitution is required, this rule should be applied, the form taken being as in the names of the following naturally occurring substances: β-phenyl-L-alanine, 4-hydroxy-L-proline, 3:5-diiodo-L-tyrosine, 3:5-dibromo-L-tyrosine, δ-hydroxy-L-lysine. However, in general biochemical writing, the names of the following four substances, the position of substitution of which is well understood, are admissible as exceptions: L-phenylalanine, L-hydroxyproline, L-diiodotyrosine, and L-hydroxylysine.

Addendum: Physico-chemical examination of polysaccharide A
By J. M. Creeth and B. R. Record
Volume 52 (1952), No. 1

p. 31, col. 2, line 3: *for* 0·11 *read* 0·08
p. 31, col. 2, line 1 under Table 1: *for* 4·9% *read* 3·5%

The estimation of serum bilirubin
By J. Patterson, J. Swale and Cora Maggs
Volume 52 (1952), No. 1

p. 100, col. 2, line 25: *for* sulphate *read* sulphamate

The biosynthesis of protein. 1
By P. N. Campbell and T. S. Work
Volume 52 (1952), No. 2

p. 220, col. 1, line 7: *for* 1 ml. *and* 9 ml. *read* 2 ml. *and* 8 ml.

Anaerobic and aerobic glycolysis in lactating mammary gland and in nervous tissue
By C. Terner
Volume 52 (1952), No. 2

p. 229, col. 2, lines 6 and 7:
for dry weight, determination *read* dry weight determination
p. 236, col. 1, line 7 from bottom: *delete* in accelerating

Some biochemically relevant properties of *N*-substituted fructosamines derived from amino-acids and *N*-arylglucosylamines
By A. Gottschalk
Volume 52 (1952), No. 3

p. 455, Title: *for* amino-acids and *N*-arylglucosylamines
read *N*-glucosylamino-acids and *N*-arylglucosylamines

Incorporation of [*carboxy*-^{14}C]acetate into lactose and glycerol by the lactating goat udder
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Rules 6 and 7, as adopted by the International Commission, differ somewhat from those previously published in the *Biochem. J.* The new international rules are as follows:

AMINO-ACID NOMENCLATURE

Rules 6 and 7

Final version adopted by the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry in September 1951

Rule 6. (*a*) Where the amino-acid has two centres of asymmetry so constituted that internal compensation cannot occur, two diastereomeric forms are possible both of which possess the L$_s$ configuration at the α-carbon atom; of these forms the *first to be described* is designated the L-amino-acid and its enantiomorph is designated the D-amino-acid. Where the name contains one or more prefixes denoting substitution, the L- or D- is, in accordance with Rule 7, placed immediately before the part of the name (usually the trivial name of the parent amino-acid) which signifies an asymmetric configuration around the α-carbon atom.

Examples: L-threonine, L-isoleucine, for the amino-acids of protein origin which have trivial names; D-threonine, D-isoleucine for their enantiomorphs. Where there is a prefix denoting substitution, the names take the form hydroxy-L-proline, hydroxy-DL-glutamic acid, hydroxy-L-lysine, or, with specification of position of substitution, 4-hydroxy-L-proline, β-hydroxy-DL-glutamic acid, δ-hydroxy-L-lysine.

(*b*) The other diastereomer which possesses the L$_s$ configuration at the α-carbon atom is distinguished by the prefix *allo* in addition to the prefix L. Its enantiomorph is denoted by the prefix *allo* and the prefix D. Where the name is wholly trivial, the L or D is placed before the prefix *allo* which is, in turn, attached to the parent name; but in names which contain one or more prefixes denoting substitution, the *allo*- is placed before this prefix and the L- or D- is placed as stated in section (*a*).

Examples: L-*allo*threonine, D-*allo*threonine, L-*allo*isoleucine, D-*allo*isoleucine for the pairs of enantiomorphs of the diastereomers of the amino-acids of protein origin which have trivial names; *allo*hydroxy-L-proline, *allo*hydroxy-D-proline, *allo*hydroxy-L-lysine, *allo*hydroxy-D-lysine, and similarly for the enantiomorphs of the diastereomers named as derivatives of amino-acids which have trivial names. Where the position of the substituent group is designated the names take the form *allo*-4-hydroxy-L-proline, *allo*-δ-hydroxy-L-lysine. Systematic names take the form *allo*-β-hydroxy-L-α-aminobutyric acid (for L-*allo*threonine), *allo*-4-hydroxy-L-2-pyrrolidine-carboxylic acid (for *allo*hydroxy-L-proline).

(*c*) For diastereomeric α-amino-acids which have structures not encountered in nature but which are named as derivatives of naturally occurring amino-acids with trivial names, choice between the prefixes L and D is made, (1) from the results of direct correlation with substances of known configuration, or (2) tentatively, from the results of studies of biological properties, or of the change in optical rotation with change in the conditions of observation, or both. The assignment of the prefix *allo* to the pair of enantiomorphs of one of the diastereomers is made tentatively, if possible, in accordance with the principle in section (*a*). The prefixes are placed as stated in section (*a*).

Examples: Of the four theoretically possible optically active isomers of hydroxyaspartic acid, two should yield L-aspartic acid on reduction and are accordingly hydroxy-L-aspartic acid and *allo*hydroxy-L-aspartic acid, the choice of these designations being made tentatively as specified. The respective enantiomorphs are named with the prefix D.

Rule 7. Salts and derivatives of amino-acids including peptides are designated with the use of a *small capital letter* to denote the configurational family of the *α-carbon atom* or *atoms*, this letter being placed immediately before the name of the parent acid or its radical. The customary rules of nomenclature are otherwise observed.

Examples: L-histidine monohydrochloride monohydrate, copper L-aspartate, D-ornithine dihydrochloride, acetyl-L-tryptophan, diethyl D-glutamate, β-hydroxy-DL-glutamic acid, L-leucyl-L-valine, glycyl-DL-leucine. Names in which the prefixes involve amino-acid configurations are treated similarly; thus, *S*-(D-2-amino-2-carboxyethyl)-D-homocysteine for D-cystathionine.

Where the name contains one or more prefixes denoting substitution and where specification both of configuration and of position of substitution is required, this rule should be applied, the form taken being as in the names of the following naturally occurring substances: β-phenyl-L-alanine, 4-hydroxy-L-proline, 3:5-diiodo-L-tyrosine, 3:5-dibromo-L-tyrosine, δ-hydroxy-L-lysine. However, in general biochemical writing, the names of the following four substances, the position of substitution of which is well understood, are admissible as exceptions: L-phenylalanine, L-hydroxyproline, L-diiodotyrosine, and L-hydroxylysine.

The International Commission has adopted a new Rule 10 on amino-acid radicals. This rule states that amino-acid radicals shall be named in conformity with the Definitive Report of the Commission on Nomenclature in Organic Chemistry. The rule gives a full list of amino-acid radicals with their formulae. It seems unnecessary to reproduce the whole of this list. The general rule is that for an amino-acid $R.CHNH_2.COOH$ (name ending in 'ine') the radical $R.CHNH_2.CO—$ has the corresponding name ending in 'yl'; thus 'alanine' gives the radical 'alanyl'. 'Tryptophan' forms 'tryptophyl'.

The following may be noted specially:

Asparaginyl $H_2N—CO—CH_2—CH(NH_2)—CO—$

Aspartyl $HOOC—CH_2—CH(NH_2)—CO—$

Similarly, glutaminyl, glutamyl.

Cystyl $S—CH_2—CH(NH_2)—CO—$

$S—CH_2—CH(NH_2)—CO—$

Cysteinyl $HS—CH_2—CH(NH_2)—CO—$

VITAMINS

The following names have been adopted by the Commission for the Reform of Nomenclature in Biological Chemistry of IUPAC.

Present name	Name adopted
Vitamin D_2 or calciferol	Ergocalciferol[1]
Vitamin D_3	Cholecalciferol[1]
Vitamins E	α, β- and γ-Tocopherol
Vitamin B_1, aneurin or thiamine	Thiamine
Vitamin B_2 or riboflavin	Riboflavin
Vitamin PP, niacinamide or nicotinamide	Nicotinamide
Vitamins possessed of B_{12} activity	Cobalamin[2] (collective name)
Vitamin B_{12}	Cyanocobalamin[2]
Vitamin B_{12b}	Hydroxocobalamin[2]
Vitamin B_{12c}	Nitrosocobalamin[2]
Vitamin C or ascorbic acid	Ascorbic acid

[1] These names have the advantage of permitting other D vitamins, derived from 7-dehydrosterols, to be named by analogy through the use of the appropriate prefix.

[2] These names are recommended for adoption because they conform with Werner's nomenclature for cobalt co-ordination complexes, and because they provide a system of terminology which may be extended, by analogy, to many modified cobalamins.

The names pantothenic acid, biotin, *p*-aminobenzoic acid and choline remain unchanged.

Names for the vitamins A and K, the pyridoxin group, inositol and the folic acid group are under discussion.

The Relation between Pyridoxin and Tryptophan Metabolism, Studied in the Rat

By C. E. DALGLIESH
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 28 *December* 1951)

It was long considered that the first product of tryptophan degradation in animals was hydroxytryptophan. This hypothesis rested on indirect evidence (for details and references see Dalgliesh, 1951) and it was not till synthetic hydroxytryptophan became available (Cornforth, Cornforth, Dalgliesh & Neuberger, 1951; Kotake, Sakan & Miwa, 1950) that direct evidence could be obtained. The problem was investigated in this laboratory by the feeding of supplementary tryptophan or hydroxytryptophan to normal and pyridoxin-deficient rats and subsequent chromatographic examination of the urines. As already reported (Dalgliesh, Knox & Neuberger, 1951) the complete difference in the urinary pictures obtained after feeding hydroxytryptophan (which actually exists in its tautomeric form and is more correctly described as oxindolylalanine; Cornforth, Dalgliesh & Neuberger, 1951) showed clearly that it is not a normal tryptophan metabolite. This result has now been confirmed by many workers (cf. Dalgliesh, 1951).

These investigations also showed that the urines of pyridoxin-deficient rats fed supplementary tryptophan contained many substances showing a powerful fluorescence in ultraviolet light, which did not occur either in the urine of normal rats with or without a tryptophan supplement in the diet, or in the urine of pyridoxin-deficient rats receiving no tryptophan supplement. These substances therefore represent tryptophan metabolites formed or accumulated as a result of the pyridoxin deficiency and their identification should throw light on both tryptophan metabolism and the function of pyridoxin. This paper describes a qualitative investigation of these substances.

EXPERIMENTAL AND RESULTS

Animals and diet

The rats used were Institute stock strain. From the age of 4 weeks they were fed a diet consisting of: casein (unextracted), 10%; fat mixture, 10%; sucrose, 36%; starch, 40%; and salt mixture, 4%. The fat mixture consisted of arachis oil, 80%, and cod liver oil, 20%. The salt mixture consisted of: NaCl, 22 g.; hydrated calcium phosphate, 130 g.; potassium citrate, 125 g.; $MgSO_4.7H_2O$, 30 g.; ferric citrate, 5 g.; trace mixture, 0·7 g. The trace mixture consisted of: KI, 12 g.; NaF, 10 g.; anhydrous $MnSO_4$, 2 g.; Cu_2I_2, 1 g.; anhydrous potassium alum, 1 g. To each kg. of the above diet was added a vitamin mixture (cf. Reid, Lepkovsky, Bonner & Tatum, 1944) composed of: thiamine, 10 mg.; riboflavin, 10 mg.; calcium pantothenate, 40 mg.; choline, 500 mg.; nicotinic acid, 100 mg.; *p*-aminobenzoic acid, 600 mg.; inositol, 1 g.; biotin, 25 μg.; vitamin K, 20 μg.; α-tocopherol, 10 mg.

On this regime the rats continued to grow, but only slowly. Their mean weights were: initially, 35 g.; after 3 months, 91 g.; after 6 months, 124 g.; after 9 months, 162 g.; after 13 months, 171 g. Out of seven rats of the original litter, two were killed after 5·5 months and two after 12 months when they developed acute symptoms of acrodynia. After 13 months the remaining animals were lively and well, and the same applied after 8 months to two animals subsequently placed on the diet.

The continuous, though slow, growth suggests that the diet was low in pyridoxin rather than completely deficient. The rats were in general able to adapt themselves to this low intake, which may have been derived from the casein (unextracted) or the intestinal bacteria. The results of the present work show that the amount of available pyridoxin was insufficient to enable the animals to deal normally with supplementary tryptophan in the diet. There were no apparent differences between animals which received a tryptophan supplement frequently and those which received it rarely, nor were there obvious differences between the urinary pictures of animals which developed acrodynia and those which remained well.

The first feeding experiments were carried out after 3 weeks on the diet. Supplementary tryptophan was administered by mixing with the diet at the rate 0·1 g. of L-tryptophan or 0·2 g. DL-tryptophan/rat/day for 1 or 2 days, the urine being collected for the subsequent 48 hr. The greater part of the work was done using L-tryptophan, but the same fluorescent substances were obtained after feeding the DL-isomers.

Paper chromatography

In the preliminary experiments the ascending technique (Williams & Kirby, 1948) on Whatman no. 1 paper was used at room temperature. It became clear that resolution at low R_F values was not being obtained and subsequently the descending technique (Consden, Gordon & Martin, 1944) on Whatman no. 4 paper was used, the solvent front being allowed to travel about 60 cm. The mean R_F values by the two techniques differed slightly, but individual variations between chromatograms were rendered unimportant by running reference substances.

Many solvent systems were examined. Those principally used were: phenol/water or phenol/ammonia as described by Consden *et al.* (1944); 'collidine', a 1:1 mixture of 2:4:6-

collidine and 2:4-lutidine saturated with 2 vol. of water (Dent, 1948); and the organic phase from a freshly prepared mixture of butanol (4 parts), acetic acid (1 part) and water (5 parts) (Partridge, 1946). Of these the last was by far the best in that it gave almost complete resolution of the fluorescent substances on one-dimensional chromatograms. It will be referred to in this paper as butanol/acetic acid.

Diagnostic reactions

Five properties or reactions were used for the routine examination of the chromatograms: (1) fluorescence under ultraviolet light, (2) the ninhydrin reaction, (3) Ehrlich's reaction, (4) Ekman's reaction and (5) Pauly's reaction. In addition various special reactions were applied as occasion demanded. The scope of these reactions, as applied to tryptophan metabolites and related compounds, was investigated, with the following results:

(1) *Fluorescence under ultraviolet light.* The light source was a Hanovia fluorescence lamp, model XI, fitted with a Wood's glass filter of which the principal transmission was around 3660 A., with very low transmission in the visible. Under the conditions of chromatography used in this work negligible fluorescence was shown by ordinary amino-acids (including tryptophan), tryptamine, *p*-aminobenzoic acid, nicotinic acid or amide, and quinolinic acid. On the other hand, many known tryptophan metabolites and related substances showed powerful fluorescence with considerable variation in colour, and these are summarized in Table 1. Kynurenic acid after running in either butanol/acetic acid or collidine leaves a weak blue fluorescent spot on the origin. In the absence of reference standards the actual description of a colour in this and other colour tests must be regarded to some extent as subjective.

(2) *The ninhydrin reaction.* This was carried out in the usual way. The only point calling for comment is the orange colour given by certain *N'*-acylated kynurenines and derivatives of α-amino-β-benzoylpropionic acid, discussed later in the paper.

(3) *Ehrlich's reaction.* This is of particular value in that it reveals both the indole type of structure, and also amino compounds. Moreover, the colours obtained with different types of structure vary widely and characteristically, as do the times taken for the colours to appear.

Table 1. *Fluorescence on paper of some reference substances under ultraviolet light*

Substance	Colour of fluorescence
Kynurenine	Strong pale blue
*N*α-Acetylkynurenine	Strong pale blue
o-Aminoacetophenone	Strong pale blue
N'-Formylkynurenine	Weak medium blue
N'-Acetylkynurenine	Weak medium blue
3-Hydroxykynurenine	Green
Kynurenic acid	Weak dark blue
Xanthurenic acid	Strong blue
Anthranilic acid	Purple
Acetylanthranilic acid	Purple
3-Hydroxyanthranilic acid	Blue-purple

After running in an acid or neutral solvent, the reagent was applied as a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde in 5% HCl, and the chromatogram was allowed to hang at room temperature. After running in a basic solvent difficult to remove, such as collidine, it was found advisable either to increase the concentration of HCl in the spraying reagent or to expose the chromatogram to HCl fumes prior to spraying, in order to avoid formation of an objectionable pink background.

Table 2. *Colours produced on paper by reaction between reference substances and Ehrlich's reagent*

Substance	Immediate colour	Colour after 2 hr.	Colour after 24 hr.
Tryptophan	—	Violet	Green
*N*α-Acetyltryptophan	—	Blue	Blue
Indole	Violet	Carmine	Carmine
Skatole	Violet after about 2 min.	Violet	Blue
Indolylpropionic acid	—	Blue	Blue
Indolylacetic acid	—	Blue	Blue
Tryptamine	—	Violet	Grey-violet
Oxindolylalanine	Very pale yellow	Pale yellow	Pale yellow
Oxindole	—	Pale yellow	Pale yellow
Kynurenine	Orange	Orange	Orange
*N*α-Acetylkynurenine	Orange	Orange	Orange
o-Aminoacetophenone	Orange	Orange	Orange
3-Hydroxykynurenine	Orange pink (slight delay in appearance)	Pink	Pink
Anthranilic acid	Deep yellow	Deep yellow	Deep yellow
Acetylanthranilic acid	—	—	Yellow
3-Hydroxyanthranilic acid	Very pale yellow	Pale yellow	Orange
Aniline	Deep yellow	Deep yellow	Deep yellow
p-Aminobenzoic acid	Deep yellow	Deep yellow	Deep yellow
Aliphatic amino-acids and amines	—	—	Pale yellow after some days
Urea	Bright yellow	Bright yellow	Bright yellow
Allantoin	Weak pale yellow	Pale yellow	Pale yellow

The results obtained are summarized in Table 2. In general, indole derivatives give violet or blue colours appearing only after a delay and sometimes changing in colour later. Derivatives of *o*-aminoacetophenone with an unsubstituted amino group give an orange colour immediately, whereas 3-hydroxykynurenine gives a pink (or orange-pink) colour more slowly. Aromatic amines give an immediate deep-yellow colour, whereas aliphatic amines give very pale yellow colours only visible after a long delay.

Urea gives an immediate strong bright yellow colour (cf. citrulline: Dent, 1948), and should therefore as far as possible be removed from urine preparations.

(4) *Ekman's reaction.* Aromatic amino groups were revealed by spraying the chromatogram, after diazotization, with ethyl-1-naphthylamine as a coupling reagent (Ekman, 1948; Hellmann, 1951) and this will be referred to subsequently as Ekman's reaction. Both Ekman and Hellmann carried out diazotization by spraying with dilute $NaNO_2$ in HCl. In the present work diazotization was carried out by exposing the lightly damped paper to nitrous fumes generated in a beaker from $NaNO_2$ and HCl. Aromatic amines give a magenta colour increasing in intensity on standing, but the reaction also gives much additional information. On exposure to nitrous fumes (and before spraying with the coupling reagent) tryptophan immediately gives a yellow colour, becoming browner on standing, which is presumably due to formation of the nitroso derivative. A yellow colour is also given by *o*-aminophenols such as 3-hydroxykynurenine and 3-hydroxyanthranilic acid, and after spraying with the coupling reagent and allowing to stand the colour becomes a deep brown. The initial yellow colour is in this case presumably due to benzoxdiazole formation.

(5) *Pauly's reaction.* This was carried out in the usual way. In the presence of excess nitrite tryptophan gave a yellow spot becoming brown, as with Ekman's reagent. *o*-Aminophenols gave a brown spot increasing in intensity on standing.

(6) *Ammoniacal silver nitrate.* *o*-Aminophenol derivatives such as 3-hydroxykynurenine and 3-hydroxyanthranilic acid gave a brown-black spot immediately on spraying. On being allowed to dry at room temperature red-brown spots also appeared with kynurenine, *o*-aminoacetophenone, xanthurenic acid, 8-hydroxyquinoline, and tryptophan.

(7) *Phosphate reagent.* (Hanes & Isherwood, 1949.) Comparison of radioautographs of chromatograms carrying ^{32}P with 'phosphate' spots as revealed by this reagent showed that artifacts can occur. In particular it was found that after spraying with the perchloric-molybdate solution, and heating, tryptophan gives a blue spot (before H_2S treatment).

Behaviour of reference substances

The fluorescence and behaviour to various reagents of many of these substances are described above. Below are summarized some R_F values not otherwise mentioned in the text, together with certain comments on reactivity, etc. The R_F values should be taken as approximate, as many depend on single or a few determinations where this was adequate to show by direct comparison non-identity with one of the metabolites. Butanol/acetic acid is abbreviated as B/A; collidine as C; ascending chromatograms as (a), and descending as (d).

(*A*) *Derivatives of indole:* N^{α}-Acetyltryptophan: B/A (a), 0·91; C(d), 0·70. Indolylpropionic acid: B/A(a), 0·93; C(d), 0·84. Indolylacetic acid: C(d), 0·69. Tryptamine: B/A(d), 0·66; C(d), 0·70 (weak) and 0·78 (strong). Skatole: C(d), 0·96. Indole: B/A(a), 0·98; C(d), 0·93.

(*B*) *Derivatives of o-aminoacetophenone:* DL-Kynurenine and DL-3-hydroxykynurenine are both resolved on paper chromatograms. In both cases the R_F value of the D-isomer is about 0·9 times that of the L-isomer (Dalgliesh, 1952). In a B/A(d) chromatogram a solvent run of 60 cm. results in complete separation of the kynurenine isomers, but hydroxykynurenine, with a lower R_F, may appear as a dumb-bell shaped spot.

N'-Formylkynurenine (B/A(d), 0·41) and *N'*-acetylkynurenine (B/A(d), 0·49) give an orange colour with Ehrlich's reagent only after some while, when hydrolysis of the acyl group has presumably occurred. Both give an orange colour with ninhydrin, as does also a precursor in kynurenine synthesis, α-amino-β-*o*-nitrobenzoylpropionic acid. Neither are resolved on paper chromatograms, nor is N^{α}-acetylkynurenine. *o*-Aminoacetophenone moves with or close to the solvent front.

(*C*) *Derivatives of anthranilic acid and miscellaneous substances:* Anthranilic acid: B/A(d), 0·93; C(d), 0·76. 3-Hydroxyanthranilic acid: B/A(a), 0·89; C(d), 0·84. Acetylanthranilic acid: B/A(a), 0·93; C(d), 0·80. Urea: B/A(a), 0·50; C(d), 0·52; butanol/water, 0·29. Allantoin: B/A(d), 0·30; C(d), 0·60. *p*-Aminobenzoic acid: B/A(d), 0·79; C(d), 0·69.

Preliminary experiments

The metabolite mixture readily became oxidized, and a rapid isolation procedure was therefore sought. In the first experiments the urine was submitted to the electrolytic desalting procedure of Consden, Gordon & Martin (1947), and after concentration was examined by paper chromatography. In butanol/acetic acid five strongly fluorescent spots were observed under ultraviolet light. Two of these spots appeared at medium R_F and showed a green and a strong pale-blue fluorescence, and another pair of spots with similar fluorescence appeared at high R_F. The spots of medium R_F were recognized, as described later, as L-3-hydroxykynurenine and L-kynurenine, and in Table 3 some R_F values of the metabolites (described as *E* and *F*) are compared with those of authentic materials. Tryptophan also appeared on the chromatograms, and could be readily demonstrated with Ehrlich's reagent and subsequent exposure to HCl fumes as described by Tabone, Robert, Thomassey & Mamounas (1950).

Table 3. *R_F values of some metabolites and reference substances*

Substance	R_F in butanol/acetic acid, ascending†	R_F in collidine, descending*	R_F in butanol/water, ascending†
Metabolite *E*	0·39	0·59	0·18
3-Hydroxy-L-kynurenine	0·39	0·60	0·18
Metabolite *F*	0·48	0·51	0·23
L-Kynurenine	0·48	0·51	0·23

* Whatman no. 4 paper.
† Whatman no. 1 paper.

Electrolytic desalting of the urine was not satisfactory. The darkening during treatment showed that degradation occurred, the proportions of the metabolites varied with the duration of the experiment, and relatively large amounts of non-fluorescent ninhydrin-positive substances remained, together with urea. The latter gives a powerful Ehrlich's reaction and obscures an important region of the chromatograms.

Ether and butanol extraction of the urines. Urine adjusted to pH 3 was extracted with ether, giving small amounts of the faster-moving fluorescent metabolites. Continuous extraction for some hours gave larger amounts of material, but also much urea. The urines were therefore adjusted to pH 7, treated with urease at 37°, readjusted to pH 3–4, and extracted as before. The procedure was reasonably satisfactory, but much more time-consuming than precipitation procedures. Butanol was unsuitable for extractions due to simultaneous extraction of appreciable amounts of salts.

complete resolution of these substances. In the remainder of this paper this solvent mixture is assumed to be used unless otherwise stated.

It was then found that much superior results could be obtained more simply by precipitation with mercuric acetate in acid solution, and the following procedure was adopted: the urines were diluted with an equal volume of 50% (v/v) acetic acid, and allowed to stand at 0° for 1–1·5 hr. The precipitated material, which besides protein and extraneous matter contained appreciable amounts of kynurenic and xanthurenic acids, was spun down, and to the supernatant

Table 4. *Summary of colour reactions obtained after descending chromatography in butanol/acetic acid on Whatman no. 4 paper*

(Spots down to *J* represent tryptophan metabolites. For remainder see text. A dash represents absence of colour.)

Spot	Mean R_F	Fluorescence under ultraviolet illumination	Ninhydrin	Ehrlich's reagent	Ekman's reagent	Pauly's reagent	Probable identity
A	0·07	Blue-green	Reddish purple	Pink after 1 hr. or more	Yellow on diazotizing turning brown slowly	Brown	Glycuronide of 3-hydroxy-L-kynurenine
B	0·12	Blue-purple	—	—	—	—	
C	0·15	Blue-green	Reddish purple	Pink after 1 hr. or more	Yellow on diazotizing turning brown slowly	Brown	Sulphate of 3-hydroxy-L-kynurenine
E	0·35	Green	Brown-purple	Pink after a few min.	Yellow on diazotizing turning brown slowly	Brown	3-Hydroxy-L-kynurenine
F	0·42	Strong pale blue	Purple	Immediate orange	Magenta after short delay	—	L-Kynurenine
X	0·47	Blue-purple	—	—	—	—	
G	0·52	Weaker pale blue	—	—	—	—	
H	0·65	Blue-purple	—	—	—	Red	Kynurenic and xanthurenic acids
I	0·77	Green	—	Pink after a few min.	Yellow on diazotizing turning brown slowly	Brown	N^{α}-Acetyl-3-hydroxy-L-kynurenine
J	0·82	Strong pale blue	—	Immediate orange	Magenta after short delay	—	N^{α}-Acetyl-L-kynurenine
K	0·89	Blue-purple	—	Immediate yellow	Magenta rapidly	—	
Tryptophan	0·49	—	Grey-purple	Violet after 1 hr.	Yellow on diazotizing	Yellow-brown	
*Eh*1	0·27	—	—	Immediate yellow	Magenta	—	
*Eh*2	0·61	—	—	Immediate yellow	Magenta	Brown	

Mercury precipitation of the urines. The conditions first tried were those described by Cole (1942*a*) for the precipitation of tryptophan. The urines of normal rats with or without a tryptophan supplement, and of pyridoxin-deficient rats without a tryptophan supplement, gave very slight precipitates under those conditions, whereas the urines of pyridoxin-deficient rats fed tryptophan gave a copious precipitate. The final yellow-green solution was found on chromatography to contain a wealth of fluorescent substances which were labelled consecutively from *A* to *J* (*D* being a region of weak fluorescent background without outstanding spots). Butanol/acetic acid was by far the best mixture for chromatography and gave complete or almost

was added an excess of mercuric acetate in 25% (v/v) acetic acid. A copious yellow-green or green precipitate was formed, which was spun down after 1·5–2 hr. The supernatant, if left for 24 hr., deposited further material. The composition of this was found to be qualitatively similar to that originally precipitated, and as only about 10% more material was obtained on the longer precipitation the supernatant was normally discarded. The residue was suspended in water and treated with H_2S for 1–2 hr. until the relatively slow precipitation of HgS was complete. Excess H_2S was removed by bubbling N_2 through the mixture, and after centrifugation the yellow-green supernatant was filtered, and concentrated *in vacuo*.

The material contained all the spots *A–J* obtained by the $HgSO_4$ precipitation procedure and also contained: (1) an additional fluorescent substance, *X*, running rather faster than kynurenine, (2) appreciable amounts of non-fluorescent ninhydrin-positive material, (3) urea and (4) in later experiments another fast-running fluorescent substance, *K*. The properties of the various substances are summarized in Table 4. Spots *B* and *C* tended to overlap, and where this occurred the fluorescence of the mixed spot resembled that of kynurenine. Overlapping was sometimes also observed between *F* and *X* and between *J* and *K*. Tryptophan also underlies *X* but does not fluoresce.

It should be noted that some chromogenic substances do occur in the urine fractions of control normal and pyridoxin-deficient rats isolated by the above procedure, but these differ from substances *A–J* and are in general present in comparatively small quantities. One substance of interest was, however, noted in the urines of normal rats receiving supplementary tryptophan. This gave a violet-blue colour with Ehrlich's reagent after 1–2 hr., but was not fluorescent and gave no ninhydrin reaction. It was readily extracted by ether from urine at pH 4·5, was extracted from the ether by bicarbonate, and could be re-extracted into ether on acidification. It had an R_F value of 0·90 in butanol/acetic acid (ascending) and 0·71 in collidine (descending) and appeared to be a simple tryptophan derivative less hydrophilic than tryptophan itself. Its behaviour closely resembled that of N^{α}-acetyltryptophan, but the two did not appear to be identical. The substance may be the same as the ether-soluble metabolite of Sarett & Goldsmith (1949).

Spectroscopic investigations

The individual substances were eluted from the chromatograms with 0·02 M phosphate buffer, pH 7·3, and their spectra determined between 230 and 380 mμ. using a Beckman spectrophotometer, model DU.

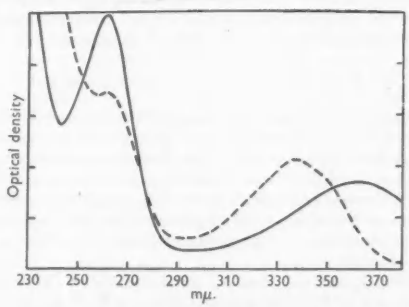


Fig. 1. Absorption spectra of substances *B* (– – –), and *C* (——) after elution from the chromatograms with buffer solution.

Fig. 1 shows the spectra of substances *B* (broken line) and *C* (full line). The spectrum of *C* is typical of that given by six of the fluorescent substances, *A*, *C*, *E*, *F*, *I* and *J*, and the positions of the maxima observed for these substances are shown in Table 5. It was observed, however, that the further the substance travelled on the chromatogram, the lower the second maximum appeared. This suggested that interaction with paper constituents was occurring. As *F* was already known to be kynurenine, authentic kynurenine was examined on chromatograms and the same depression of the second maximum, with movement to slightly lower wavelength, was observed. Interaction with paper constituents is also indicated by the resolution observed for DL-kynurenine and DL-hydroxykynurenine, a result which could not occur if the chromatography only involved partition between the optically inactive solvent phases. The ready formation of a complex between L-kynurenine and sucrose, used for the preparative resolution of kynurenine (Butenandt & Weichert, 1944) is also relevant in this connexion. It will be noticed that the second maxima of *A*, *C*, *E* and *I* lie at about 365 mμ., whereas those of *F* and *J* lie at about 355 mμ. The first four substances also appear pale yellow on the untreated chromatogram.

Table 5. *Absorption maxima of eluted substances*

Substance	Position of first maximum (mμ.)	Position of second maximum (mμ.)
A	264	364
C	262	362·5
E	264	368
F	263	355
I	265	365
J	265	355

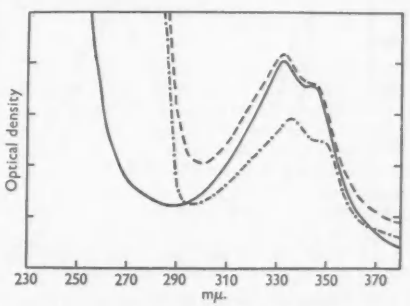


Fig. 2. Absorption spectra of eluted substances *X* (–·–·–) and *H* (– – –) compared with a 1:1 mixture of kynurenic and xanthurenic acids (——).

Fig. 2 shows the spectra of substances *X* and *H* and the general resemblance between these and the calculated spectrum of a 1:1 mixture of kynurenic and xanthurenic acids. Both *X* and *H* appear to absorb strongly below 290 mμ. In the case of *X* this is known to be due to the presence of tryptophan, which underlies *X* on the chromatogram, and it is probable that other indole-type compounds are responsible for the similar absorption of *H*.

The significance of these spectra is considered in detail when the identity of the individual substances is discussed. At the same time various known compounds were examined spectroscopically, and the following are the results obtained ('buffer' refers to the mixture already described): *Kynurenine:* in buffer showed maxima at 257 mμ. (ϵ=6750) and 361 mμ. (ϵ=4350) (Fig. 3). *3-Hydroxykynurenine:* in buffer showed maxima at 268·5 mμ. (ϵ=9840) and 370 mμ. (ϵ=4240). In water the peaks were unchanged in position

and had ϵ values of 7800 and 4000 respectively (Fig. 3). In 0·1N-HCl the maximum at 370 mμ. had almost disappeared (ϵ = 740) and a new maximum appeared at 315 mμ. (ϵ = 2650); the lower wavelength maximum had moved to 251·5 mμ. (ϵ = 8750) (Fig. 3). *Kynurenic acid:* in buffer showed maxima at 332 mμ. (ϵ = 9800) and 344 mμ. (ϵ = 7920) (Fig. 4). In 0·1N-HCl the specimen was too insoluble to determine

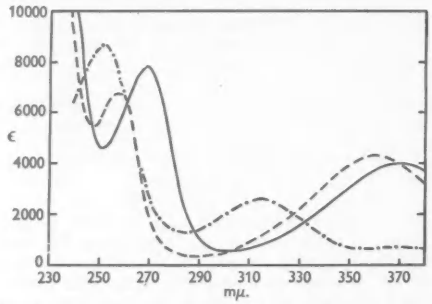


Fig. 3. Absorption spectra of 3-hydroxykynurenine in water (——), kynurenine in buffer (– – –) and 3-hydroxykynurenine in 0·1N-HCl (–·–·–).

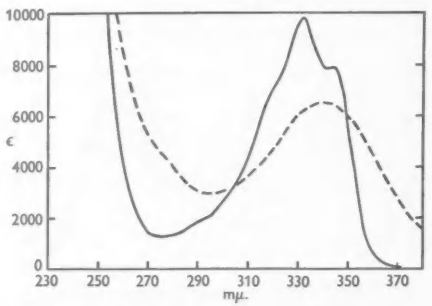


Fig. 4. Absorption spectra of kynurenic acid (——), and xanthurenic acid (– – –), in buffer.

accurate ϵ values, but showed a maximum at 310 mμ. descending to a flat area centred on 325 mμ. In 0·1N-NaOH the twin peaks had coalesced to one flat maximum from 320–325 mμ. (ϵ = 6350). *Xanthurenic acid:* in buffer showed a wide maximum centred at 340 mμ. (ϵ = 6500) (Fig. 4). In 0·1N-HCl ϵ values were again not determined due to insolubility, but the spectrum showed an inflexion at 315mμ. and a maximum at 353 mμ. superimposed on appreciable general absorption. In 0·1N-NaOH maxima at 282 mμ. (ϵ = 5300) and 327 mμ. (ϵ = 5300) were again superimposed on general absorption. *Anthranilic acid:* in buffer showed maxima at 241 mμ. (ϵ = 6400) and 309 mμ. (ϵ = 2900). *3-Hydroxyanthranilic acid:* in buffer showed an inflexion at about 240 mμ. and a maximum at 314 mμ. (ϵ = 3240). In 0·1N-HCl showed a maximum at 297 mμ. (ϵ = 3120), agreeing with the results of Nyc & Mitchell (1948).

Effect of autoxidation on the spectra. The spectra of hydroxykynurenine and hydroxyanthranilic acid were examined at varying times after making up the solutions to see whether oxidation during isolation and the chromatographic procedures might have produced changes in the spectra of the metabolites. Little change in the spectrum of a hydroxykynurenine solution was observed during the first few hours. The solution then slowly became more yellow and in the spectral region examined this was accompanied by an increase in general absorption with reduction in ϵ values, but no change took place in the position of the maxima. Thus for hydroxykynurenine at 268·5 mμ. the ϵ value of 7800 in fresh solution had dropped to 6460 after 4 days. Hydroxyanthranilic acid showed similar results.

Experiments with ^{32}P-labelled phosphate

Hanes's phosphate reagent showed the presence of a phosphate-containing substance sufficiently close to *B* and *C* to raise the question of identity. In addition, further spots were observed corresponding to tryptophan and to substance *Eh* 2. To settle this question tryptophan was fed in the usual manner, together with 0·2 ml. of a phosphate solution containing 16 μc. of ^{32}P. Chromatograms were examined by the radioautograph technique and by means of the mechanical scanning technique previously mentioned (Dalgliesh *et al.* 1951). Only one phosphorus-containing compound was observed, of R_F 0·14. The outline did not correspond exactly with that of *B* or *C*, and non-identity was conclusively proved by the subsequent observation that whereas *B* and *C* were adsorbed by deactivated charcoal, the phosphorus compound was not. The two other spots observed with Hanes's reagent were artifact spots (see above). The urinary phosphorus compound was compared with inorganic phosphate with which it was not identical (distances run on a chromatogram were 9·0 and 14·5 cm. respectively). Further experiments showed that when the urine of normal rats was submitted to the mercuric acetate precipitation procedure a phosphorus compound was isolated, whether or not supplementary tryptophan had been fed. In this case the phosphorus compound was ninhydrin-positive, thereby differing from the compound in the urine of pyridoxin-deficient rats. The substances have not been further examined.

Purification with deactivated charcoal

The work of Synge & Tiselius (1949) suggested that deactivated charcoal might be of considerable value in separating the tryptophan metabolites from contaminating substances, and this proved to be the case. Charcoal (British Drug Houses Ltd.) deactivated by 4% (w/w) stearic acid by the procedure of these authors gave excellent results, it being found unnecessary to carry out a preliminary purification of the charcoal.

The supernatant obtained by centrifugation, after precipitation of HgS and removal of excess H_2S, was shaken with the deactivated charcoal, filtered, and the residue well washed with water. The colourless filtrate contained (*a*) all the ninhydrin-positive non-fluorescent substances (presumably simple amino-acids) in the *A–E* region, (*b*) the phosphate compound in the *B–C* region, and (*c*) urea. The charcoal was then eluted with 5% (w/v) phenol and the yellow eluate concentrated in the usual way. All the fluorescent substances (and also tryptophan) were present with no apparent change in their relative proportions. Direct charcoal adsorption from the urine was also examined. After acidification with acetic acid and centrifugation in the usual way the supernatant was shaken with the

deactivated charcoal, filtered, and washed with water. The filtrate in this case was yellow. On elution with phenol as before the eluate, also yellow, was found to contain all the usual metabolites. This isolation procedure is much quicker than any other examined, but as there is some increase in background fluorescence it was preferred to use the mercuric acetate procedure followed by charcoal treatment.

Ion-exchange columns

Various resins were examined to see whether a rapid selective separation could be obtained. Zeo-Karb 225 was found satisfactory for this purpose, as it adsorbed all substances except *B* and *C*. The ionizing groups of this resin are solely of the sulphonic acid type, and substances *B* and *C* must therefore be strongly acidic. Resins relying for their activity on carboxyl groups were of no value.

B' of R_F 0·27 formed from *B* on mild hydrolysis (see below) together, surprisingly, with hydroxykynurenine, *E*. As considerable heating occurred during the experiment it seemed likely that *B'* and *E* were degradation products, and this was confirmed in an experiment in which the contents of the anode compartment were removed every 10 min. and replaced by fresh electrolyte. In this case the anode compartment contained *B* and *C* with only small amounts of *B'* and *E*. By increasing the concentration of acetic acid in the electrolyte the tendency of substances *A*, *I* and *J* to move towards the anode could be reduced and even reversed owing to the increasing part played by the positively charged form of an aromatic amino group.

To get a more complete picture of the relative mobilities of the various substances the following procedure was adopted: electrodialysis was carried out as before, using 0·01N-acetic

Table 6. *Distribution of the metabolites on electrodialysis*

Compartment	Substances present												
4			*B*	*C*	(*B'*)*	(*E*)			*G*		(*H*)		
3	4′	(*A*)	*B*	*C*	(*B'*)*	(*E*)			*G*		*H*		
	3′	(*A*)	(*B*)	(*C*)				*X*			*H*	*I*	*J*
	2′	*A*	(*B*)	(*C*)				*X*				*I*	*J*
	1′	*A*								*Eh*2		*I*	*J*
2					*Eh*1	*E*	*F*	Try.					*K*
1					(*Eh*1)	*E*	*F*	Try.					(*K*)

* *B'* represents the substance formed from *B* on mild hydrolysis (see text).
Try. = tryptophan.

Electrophoresis and electrodialysis experiments

The apparatus of Gordon, Gross, O'Connor & Pitt-Rivers (1952) was used to investigate the electrophoretic behaviour of the metabolites and known reference substances on filter-paper strips. Whatman MM paper was used, with solutions of acetic acid of varying strength as the electrolyte, and an applied potential of 240 V. The metabolites separated into four groups. Group I moved towards the cathode and kynurenine, hydroxykynurenine and tryptophan were found to travel in a similar manner. Group II moved slightly towards the cathode in 2% acetic acid but stayed close to the origin in 0·01N-acetic acid, N^{α}-acetylkynurenine behaved similarly. Group III moved towards the anode, kynurenic and xanthurenic acid behaving similarly. Group IV consisted of bands, which appeared to correspond in properties to *B* and *C*, moving fast to the anode.

In view of these favourable results electrodialysis was investigated using the apparatus and types of diaphragm described by Synge (1951). All compartments were filled with 25 ml. of the electrolyte, the specimen being in compartment 2 (cathode compartment = 1). The applied potential was 240 V., and the experiments continued till most of the colour had migrated from compartment 2. The contents of each compartment were then removed, concentrated, and examined in the usual way. Using 0·01N-acetic acid as electrolyte the cathode compartment was found to contain kynurenine (*F*), hydroxykynurenine (*E*) and tryptophan; compartment 2 contained the same substances together with *Eh*1 and *Eh*2 (see below) and *K*. Compartment 3 contained *A*, small amounts of *B* and *C*, *X* and substances *G* to *K*. The material in the anode compartment was dark in colour and had obviously undergone decomposition. The main constituents were the substance acid as electrolyte, the contents of the anode compartment being frequently replaced to reduce decomposition. When most of the colour had migrated from compartment 2 the contents of each compartment were removed and numbered 1–4. The contents of cell 3 (containing the greatest number of the metabolites) were then placed in cell 2 and the procedure repeated using this time 8% (v/v) acetic acid as electrolyte, to give a new distribution, the contents of the compartments being labelled 1′–4′. Effectively this arrangement is equivalent to an eight-compartment cell. The contents of each compartment are indicated in Table 6, brackets indicating small quantities, and underlining indicating that the greater part of that particular substance appeared in that compartment. The substances as written from left to right correspond with the downward order in which they appear on a chromatogram. It should be noted: (*a*) that the simple amino-acids kynurenine (*F*), hydroxykynurenine (*E*) and tryptophan can be fairly clearly separated from the other metabolites; (*b*) that *A*, *I* and *J* are of similar acidity and appreciably more acidic than the amino-acids; and (*c*) that *B* and *C* are strongly acidic and fairly readily hydrolysed.

Other separative procedures

Solvent extraction experiments on the crude urine preparation, attempts to use ether-buffered Celite columns, and attempted countercurrent distribution between butanol and water or butanol and 0·01N-HCl gave no useful results, though the latter demonstrated the hydrophilic nature of substances *A*, *B* and *C*.

Hydrolysis experiments

A urine preparation was heated in a sealed tube with 5N-HCl for 2 hr. at 100°. Removal of the acid and chromatography of the residue showed that substances *A*, *B*, *C*, *X*, *G*,

I and *J* had disappeared, whilst *E*, *F* and *H* remained. In further experiments the urine preparations were applied as a streak to the chromatogram and after development and drying the corresponding spots were cut out, eluted and taken to dryness. Where complete separation did not occur, the eluate was again run, this time in phenol/water, and after development and drying the spots were again cut out, eluted and concentrated. Hydrolysis was carried out under varying conditions. The following R_F values were noted in phenol/water: *A*, 0·15; *B*, 0·18; *C*, 0·31; *X*, 0·24; L-kynurenine, 0·82.

Investigation of blood plasma

A pyridoxin-deficient rat was given 0·5 g. DL-tryptophan in the diet, and next day was killed by anaesthetization and withdrawal of the blood from the heart after heparin injection. The blood (approx. 10 ml.) was diluted with an equal volume of trichloroacetic acid solution (10% w/v) and after 45 min. the mixture was centrifuged. The supernatant was precipitated with mercuric acetate in the usual way, only a slight whitish precipitate being obtained. Chromatography showed that none of the fluorescent urinary metabolites was present.

Feeding experiments with L-*kynurenine*

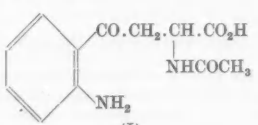
When the tryptophan supplement to the diet of pyridoxin-deficient rats was replaced by 0·1 g. L-kynurenine sulphate the urine was found to contain all the fluorescent metabolites observed after feeding L-tryptophan. This makes it most unlikely that any of these substances represent an intermediate stage between tryptophan and kynurenine.

IDENTITY OF THE METABOLITES

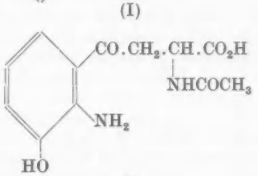
Substances E, F and H. Substances *E* and *F* showed in all respects the same behaviour as the L-isomers of 3-hydroxykynurenine and kynurenine. Thus they agreed in R_F values (e.g. Table 3), colour reactions (as in Table 4), spectroscopic behaviour, were resistant to hydrolysis and moved in the same manner on electrophoresis.

The spot *H* contains such kynurenic and xanthurenic acids as are not precipitated in the initial acid treatment of the urines. Known specimens of these two substances were found to be unresolved on chromatograms and to show the same R_F and fluorescence as *H* (the fluorescence of xanthurenic acid is more intense than that of kynurenic acid, and makes the dominant contribution). The spectrum of *H* shows the typical twin peak of kynurenic acid (Figs. 2 and 4), xanthurenic acid only having a broad maximum in this region. As is to be expected *H* is unaffected by hydrolysis. Xanthurenic acid is already known to occur in the urine of tryptophan-fed pyridoxin-deficient rats (Lepkovsky, Roboz & Haagen-Smit, 1943).

Substances I and J. The great similarity between the pair of substances *E* and *F* and the pair *I* and *J* strongly suggested that *I* and *J* were analogous derivatives of *E* and *F*. It will be seen from Table 4 that the reactions of *E* and *I* to Ehrlich's, Ekman's and Pauly's reagents were the same, as were the colours shown on fluorescence, and there was similar agreement between substances *F* and *J*. But whereas *E* and *F* gave a ninhydrin reaction, *I* and *J* did not. This suggested that *I* and *J* were derivatives of hydroxykynurenine (*E*) and kynurenine (*F*) respectively in which a substituent was attached to the aliphatic amino group. The similarity of the spectra of *E* and *I* and of *F* and *J* gave further support, as it is known (Knox & Mehler, 1950; Dalgliesh, 1952) that N^{α}-acylkynurenines have the same type of spectrum as kynurenine itself. Hydrolysis with 0·1 N-HCl at 95° for 1 hr. caused slight hydrolysis of *I* to *E*, but the greater part was unchanged, whereas hydrolysis with N-HCl for 1·5 hr. at 95° caused almost complete hydrolysis of *I* to *E*, and similarly of *J* to *F*. Moreover, both *E* and *I* gave an immediate brown-black spot with ammoniacal silver nitrate, showing that the *o*-aminophenol system was intact. It has been shown by Yanofsky & Bonner (1950) that a mutant strain of *Neurospora*, in which the further metabolism of kynurenine was impaired, accumulated a substance identified as N^{α}-acetylkynurenine (I). This was therefore synthesized (Dalgliesh, 1952) and its behaviour was found to agree with that of substance *J* in every respect.



(I)

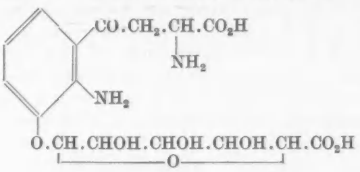


(II)

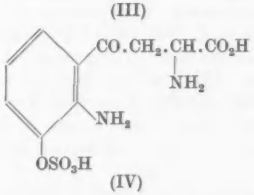
In view of the high R_F values given by *J* confirmation for this identification was desirable and was obtained, as has already been reported (Dalgliesh *et al.* 1951) by simultaneous feeding of tryptophan and sodium [$\alpha-{}^{14}C$] acetate when the activity appeared in large amounts in substances *I* and *J*. (It should be mentioned here that the lesser activity observed on the chromatograms at low R_F is likely to have been due to 'background' substances which are not tryptophan metabolites. At the time the experiment was done the purification with deactivated charcoal which removes these substances had not been worked out.) Substance *J* is therefore almost certainly N^{α}-acetyl-L-kynurenine (only L- and no D-kynurenine being formed on hydrolysis), and by analogy there is a high probability that substance *I* is N^{α}-acetyl-L-3-hydroxykynurenine

(II), though in this case synthetic material has not been available for comparison. The behaviour on electrodialysis is in accord with these formulations.

Substances A and C. Both these substances, like *E* and *I*, appear yellow on the untreated chromatogram and on hydrolysis give L-3-hydroxykynurenine. Substance *A* moves at the same speed on electrophoresis or electrodialysis as the N^{α}-acetyl compounds (I) and (II), and it is reasonable to infer that *A* also has one carboxyl group in excess of the basic groups present (neglecting the aromatic amino groups, which are of very low basicity). The ninhydrin reaction shows that the aliphatic amino group is intact and the residue conjugated with hydroxykynurenine therefore probably contains one carboxyl group (or one basic and two carboxyl groups, etc.). The very hydrophilic nature of *A*, shown both in the counter-current distributions and by its low R_F, suggests a polyhydroxy compound. The delay in appearance of a colour with Ehrlich's reagent indicates that some hydrolysis is necessary before reaction occurs. The similarity of the spectrum of *A* to that of kynurenine shows that the aromatic amino group is not acylated as *N'*-acylkynurenines have markedly different spectra (cf. Dalgliesh, 1952). An *N'*-alkylkynurenine derivative might show the type of spectrum observed, but an alkyl group would be unlikely to be lost on hydrolysis. A substance which might be expected to show the observed properties of *A* is a glycuronide such as (III). This would be expected to show greater resistance to oxidation than hydroxykynurenine, as



(III)



(IV)

is in fact observed. Thus if a chromatogram is left exposed to the air both *E* and *I*, in which the *o*-aminophenol system is intact, turn brown, whereas *A* and *C* are unchanged in colour. Moreover, unlike *E* and *I*, neither *A* nor *C* show immediate reduction of ammoniacal silver nitrate.

A striking property of substance *C* is its high acidity, shown both in the experiments with ion-exchange resins, and on electrodialysis. The acidity is such as to suggest an inorganic acid grouping to be responsible, and as the experiments with ^{32}P-labelled phosphate excluded a substituted phosphoric acid the phenolic sulphate (IV) was considered to be the most likely structure. Strong support for the formulation of *A* and *C* as (III) and (IV) respectively was obtained in hydrolysis experiments.

Hydrolysis of *A* with 0·1N-HCl for 1 hr. at 95° left it almost completely unchanged, though a trace of hydroxykynurenine was detectable. *C*, however, was almost entirely hydrolysed under these conditions, with formation of L-3-hydroxykynurenine. When a portion of the hydrolysate of *C* was tested with $BaCl_2$ a white precipitate was obtained, and no such precipitate was obtained from the hydrolysed eluate of substance *E* used as a control. This supports the formulation (IV) for *C*, which is a readily hydrolysable sulphate; no substance other than hydroxykynurenine, sulphate and a trace of unchanged *C* could be detected in the hydrolysate. Hydrolysis of *A* with N-HCl at 95° for 1·5 hr. still left the material mainly unchanged, though a somewhat larger amount of hydroxykynurenine was formed than in the previous case. After hydrolysis with 6N-HCl in a sealed tube at 100° for 1 hr. some of *A* still remained unchanged, but the greater part was hydrolysed to hydroxykynurenine. In this case a second hydrolysis product was also observed at $R_F = 0{\cdot}22$, which showed a weak blue fluorescence and gave an orange ninhydrin reaction. The same material was found to be formed from hydroxykynurenine itself under similar conditions and it is probably a substance formed by reaction of the *o*-aminophenol grouping with soluble components of the paper. It is interesting in this connexion to recall that weak blue fluorescence and an orange ninhydrin colour were found to be shown by *N'*-acylated kynurenines, and that a yellow or orange ninhydrin colour has also been reported to be given by the unsubstituted phenacylglycine, $Ph.CO.CH_2.CH(NH_2).CO_2H$ (Fraser & Raphael, 1950; Wiss, Viollier & Waldi, 1951).

These hydrolytic results are in agreement with the formulation of *A* and *C* as the *O*-glycuronide and *O*-sulphate respectively of L-3-hydroxykynurenine and may be compared with other recent results for similar phenolic derivatives (Porteous & Williams, 1949; Parke & Williams, 1951; Berenbom & Young, 1951). Confirmation for the presence of glycuronic acid was obtained by applying Tollens's reaction according to the procedure of Cole (1942*b*). Unhydrolysed *A* gave a negative test, but after hydrolysis with 5N-HCl in a sealed tube at 100° for 1 hr. the test was positive.

Substances B and X. Both *B* and *X* show a blue-purple fluorescence, that of *X* being weaker. Neither substance shows any reaction with the usual reagents and evidence on their constitution is therefore mainly derived from spectroscopic and hydrolytic data.

The spectrum of *X* (Fig. 2) has the typical twin peak of quinoline derivatives with maxima at 336 and 348 mμ., which may be compared with that of kynurenic acid (Fig. 4) showing maxima at 332 and

344 mμ. under the same solvent conditions, and kynurenine (4-hydroxyquinoline) which has maxima at 316 and 328 mμ. (Butenandt, Karlson & Zillig, 1951). Substance *X* is completely hydrolysed to kynurenic acid on heating with N-HCl for 1·5 hr. at 95°. The residue conjugated with the kynurenic acid was not further investigated.

The spectrum of *B* (Fig. 1) shows a broad maximum at 337 mμ. A similar maximum is shown by xanthurenic acid at 340 mμ. under the same solvent conditions, whereas anthranilic acid, the only other likely substance which the fluorescence at all resembles, has a maximum at 310 mμ., at which point *B* shows a minimum. (The small peak in the spectrum of *B* at about 260 mμ. is probably due to contamination by *C*.) *B* is strongly acidic, moving rapidly to the anode on electrodialysis and being unadsorbed by Zeo-Karb 225. After hydrolysis with 0·1 N-HCl for 1 hr. at 95° most of *B* is destroyed, and as with *C*, sulphate ion is detectable in the hydrolysate. However, only traces of xanthurenic acid are formed and the material is instead transformed to a substance *B′* with the same colour of fluorescence as *B* but a higher R_F (0·27). After hydrolysis with N-HCl at 95° for 1·5 hr. *B′* is still the main product, but hydrolysis with 5N-HCl in a sealed tube for 1 hr. at 100° gives xanthurenic acid. These results indicate that in *B* xanthurenic acid is conjugated with sulphate and with some other residue, which was not identified.

Substances G, K and minor components. Substances *A*, *B*, *C*, *E*, *F*, *I* and *J* are powerfully fluorescent substances which appear consistently, as do *X* and *H* though their fluorescence is less intense. Substance *G*, however, varies considerably, being fairly strong in some preparations and very weak in others. Whether this is due to variations in the urine, or in the isolation is not known. The colour of fluorescence somewhat resembles that of kynurenine, but is weaker and whiter, *G*, like *B* and *X*, does not react with the normal reagents used in this work. It was not further investigated.

Substance *K* may be a tryptophan metabolite, but its status has yet to be clarified. Urine of pyridoxin-deficient rats which had received no tryptophan supplement was found to contain a substance which had the same R_F as *K*, and gave an immediate strong yellow Ehrlich's reaction, but which was not fluorescent. The fluorescent material *K* did not appear in the urines until some time after the establishment of pyridoxin-deficiency, and as the use of DL-tryptophan started at about the same time it is possible that it is a metabolite of D-tryptophan. It is possible to separate *K* from the non-fluorescent Ehrlich-positive substance by electrodialysis, and after separation *K* also shows an immediate strong yellow colour with Ehrlich's reagent.

Substance *Eh* 1 is not present in large amounts, is non-fluorescent and occurs in the urines when no tryptophan has been fed. Substance *Eh* 2 has only been observed in any quantity after feeding DL-tryptophan, and may therefore also be a metabolite of the D-isomer. In general after feeding DL- rather than L-tryptophan 'background' substances increase, and appreciably more tryptophan (presumably mainly the D-isomer) is excreted unchanged. After feeding the DL-compound a narrow band with typical kynurenine-type fluorescence appears on the leading edge of the hydroxykynurenine spot, suggesting a small conversion of D-tryptophan to D-kynurenine. Such a transformation is known to occur in the rabbit (Kotake & Ito, 1937).

DISCUSSION

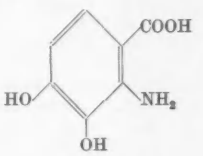
The most striking feature of the various metabolites which have been recognized is that all still contain the carbon atoms originating from the alanine side chain of tryptophan. In the normal conversion of tryptophan to nicotinic acid these carbon atoms are known from isotope experiments to be lost (Heidelberger, Gullberg, Morgan & Lepkovsky, 1949; Hundley & Bond, 1949), and this immediately suggests that pyridoxin is concerned in tryptophan metabolism at the stage at which removal of the side chain occurs. This chemical evidence agrees with evidence from enzyme studies. Kynurenine was shown by Kotake & Nakayama (1941) to be converted to anthranilic acid by an enzyme kynureninase occurring in cat liver and kidney. Subsequent work has shown that kynureninase, which equally converts 3-hydroxykynurenine to 3-hydroxyanthranilic acid, occurs in all animal species tested, and requires pyridoxal phosphate as a co-enzyme (Braunshtein, Goryachenkova & Pashkina, 1949; Wiss & Hatz, 1949; Wiss, 1949; Wiss & Fuchs, 1950; Dalgliesh *et al.* 1951). As pyridoxin is known not to affect the conversion of tryptophan to kynurenine (Braunshtein *et al.* 1949) or of 3-hydroxyanthranilic acid to nicotinic acid (Henderson, Weinstock & Ramasarma, 1951) the site of action of pyridoxin becomes clear, and the accumulation of metabolites still retaining the carbon atoms of the original tryptophan side chain gives strong support to the enzymic evidence.

The occurrence of 3-hydroxykynurenine derivatives conjugated through the phenolic group as the glycuronide (III) and sulphate (IV) indicates that the rat is dealing with an abnormally accumulated metabolite by well established detoxication mechanisms for phenolic substances. These two derivatives are therefore unlikely to be of importance in normal tryptophan metabolism. The occurrence of the N^{α}-acetyl derivatives (I) and (II) may, however, be of greater significance. Although acetyla-

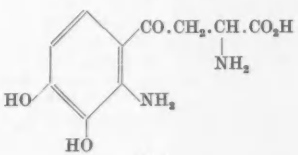
tion is a recognized detoxication mechanism, occurring, e.g., with aromatic amino groups, acetylation of the aliphatic amino group of naturally occurring amino-acids has been rarely recorded. As already pointed out (Dalgliesh, 1951) if N^{α}-acetylkynurenine were the normal substrate for hydroxylation of the aromatic ring it would be possible for the animal to direct the metabolic changes to hydroxyanthranilic acid at the expense of the formation of anthranilic and kynurenic acids which are not precursors of nicotinic acid in animals.

Certain stages in tryptophan metabolism are still obscure. The first oxidation product of tryptophan, which is in its turn further oxidized to formylkynurenine, is still unidentified. No evidence for such a compound in the urines was obtained in the present work, suggesting that oxidation to formylkynurenine is a rapid process. It is of interest that neither were appreciable amounts of formylkynurenine detected. Formylkynurenine would not be detectable as such on the chromatograms, in which it would be masked by kynurenine, but it would be detectable spectroscopically in the eluted material. Moreover, treatment of the urine preparations with formylase caused no appreciable drop in ultraviolet absorption at 320 mμ. with a corresponding rise at 360 mμ. (cf. Knox & Mehler, 1950). The result is to be expected in view of the known high activity of formylase in the intact animal.

The intermediate stages in the conversion of 3-hydroxyanthranilic acid to nicotinic acid are also still obscure. Opening of the benzene ring must occur, and it has been suggested that 3:4-dihydroxyanthranilic acid (V) forms a step in the chain



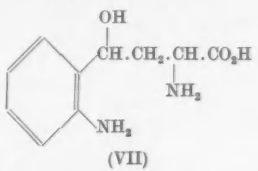
(V)



(VI)

(Makino, Itoh & Nishi, 1951). If this were the case it might by analogy be expected that (V) would be formed by the action of kynureninase on the dihydroxykynurenine (VI) and that (VI) or derivatives thereof would accumulate in the urine in pyridoxin deficiency. It would be expected that (VI) and its derivatives would be more hydrophilic than the corresponding derivatives of hydroxykynurenine, which they would otherwise resemble. No substances with the expected properties were detected, and this suggests that (VI) is not a major tryptophan metabolite. Dihydroxyanthranilic acid (V) cannot, however, be similarly excluded but it is probable that if (V) is an intermediate then introduction of the second phenolic group occurs after loss of the alanine side chain.

It seems probable that tryptophan can be hydroxylated in the benzene ring. For example, the recognition of the vasoconstrictor principle of ox serum, serotonin, as 5-hydroxytryptamine (Rapport 1949; Hamlin & Fischer, 1951) suggests that 5-hydroxylation can occur. No evidence was obtained in the present work to suggest the occurrence in any appreciable quantity of a substance such as 5-hydroxykynurenine, and this and similar pathways therefore probably play only a minor quantitative role in tryptophan metabolism. The finding of Inagaki (1933) that rabbits metabolize *o*-aminoacetophenone by oxidizing part to anthranilic acid and reducing part to *o*-aminophenylmethylcarbinol suggests the possibility of the occurrence of carbinols such as (VII) corresponding to kynurenine and hydroxykynurenine.



(VII)

But here again no evidence for compounds with the expected properties was found.

SUMMARY

1. On administration of tryptophan to rats on a low pyridoxin diet numerous metabolites appear in the urine, and the probable identity of the majority of these has been established. A convenient method for the group isolation of these substances is described, involving precipitation by mercuric acetate in acid solution and selective adsorption on deactivated charcoal.

2. The scope of various reagents, in particular Ehrlich's and Ekman's reagents, has been studied, and chromatographic, chemical, spectroscopic and some other properties of the metabolites are recorded.

3. Besides the previously known metabolites L-kynurenine, kynurenic acid and xanthurenic acid, the occurrence of L-3-hydroxykynurenine as a tryptophan metabolite has been demonstrated for the first time in animals.

4. N^{α}-Acetyl derivatives of both L-kynurenine and L-3-hydroxykynurenine are excreted and it is suggested that these compounds may be normal metabolic intermediates. In addition L-3-hydroxykynurenine is excreted with the phenol group conjugated as the glycuronide and sulphate. These are probably detoxication products.

5. The occurrence of conjugated derivatives of both kynurenic and xanthurenic acids has been demonstrated.

6. The fact that all these metabolites retain the carbon atoms of the original alanine side chain of tryptophan provides strong chemical evidence that pyridoxin participates in tryptophan metabolism at the stage at which this side chain is split from the rest of the molecule.

7. Alternative metabolic pathways for tryptophan are discussed and it is suggested that 3:4-dihydroxykynurenine is unlikely to be a tryptophan metabolite of major importance.

I should like to express my thanks to Dr A. Neuberger, F.R.S., and Dr W. E. Knox for many valuable discussions and for making available their collections of tryptophan metabolites; also to Prof. A. Butenandt for supplying synthetic hydroxykynurenine; and to Mr A. J. Tilley, Mrs B. Higginson and Miss L. Langford for technical assistance and care of the animals.

REFERENCES

Berenbom, M. & Young, L. (1951). *Biochem. J.* **49**, 165.
Braunshtein, A. E., Goryachenkova, E. V. & Pashkina, T. S. (1949). *Biokhimiya*, **14**, 163.
Butenandt, A., Karlson, P. & Zillig, W. (1951). *Hoppe-Seyl. Z.* **288**, 125.
Butenandt, A. & Weichert, R. (1944). *Hoppe-Seyl. Z.* **281**, 122.
Cole, S. W. (1942*a*). *Practical Physiological Chemistry*, p. 128, 9th ed. Cambridge: Heffer.
Cole, S. W. (1942*b*). *Practical Physiological Chemistry*, p. 322, 9th ed. Cambridge: Heffer.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Cornforth, J. W., Cornforth, R. H., Dalgliesh, C. E. & Neuberger, A. (1951). *Biochem. J.* **48**, 591.
Cornforth, J. W., Dalgliesh, C. E. & Neuberger, A. (1951). *Biochem. J.* **48**, 598.
Dalgliesh, C. E. (1951). *Quart. Rev. chem. Soc.* **5**, 227.
Dalgliesh, C. E. (1952). *J. chem. Soc.* p. 137.
Dalgliesh, C. E., Knox, W. E. & Neuberger, A. (1951). *Nature, Lond.*, **168**, 20.
Dent, C. E. (1948). *Biochem. J.* **43**, 169.
Ekman, B. (1948). *Acta. chem. scand.* **2**, 383.
Fraser, M. M. & Raphael, R. A. (1950). *J. chem. Soc.* p. 2245.
Gordon, A. H., Gross, J., O'Connor, D. & Pitt-Rivers, R. V. (1952). *Nature, Lond.*, **169**, 19.
Hamlin, K. E. & Fischer, F. E. (1951). *J. Amer. chem. Soc.* **73**, 5007.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Heidelberger, C., Gullberg, M. E., Morgan, A. F. & Lepkovsky, S. (1949). *J. biol. Chem.* **179**, 143.
Hellmann, H. (1951). *Hoppe-Seyl. Z.* **287**, 205.
Henderson, L. M., Weinstock, I. M. & Ramasarma, G. B. (1951). *J. biol. Chem.* **189**, 19.
Hundley, J. M. & Bond, H. W. (1949). *Arch. Biochem.* **21**, 313.
Inagaki, S. (1933). *Hoppe-Seyl. Z.* **214**, 25.
Knox, W. E. & Mehler, A. H. (1950). *J. biol. Chem.* **187**, 419, 431.
Kotake, Y. & Ito, N. (1937). *J. Biochem., Tokyo*, **25**, 71.
Kotake, T. & Nakayama, Y. (1941). *Hoppe-Seyl. Z.* **270**, 76.
Kotake, M., Sakan, T. & Miwa, T. (1950). *J. Amer. chem. Soc.* **72**, 5085.
Lepkovsky, S., Roboz, E. & Haagen-Smit, A. J. (1943). *J. biol. Chem.* **149**, 195.
Makino, K., Itoh, F. & Nishi, K. (1951). *Nature, Lond.*, **167**, 115.
Nyc, J. F. & Mitchell, H. K. (1948). *J. Amer. chem. Soc.*, **70**, 1847.
Parke, D. V. & Williams, R. T. (1951). *Biochem. J.* **48**, 621.
Partridge, S. M. (1946). *Nature, Lond.*, **158**, 270.
Porteous, J. W. & Williams, R. T. (1949). *Biochem. J.* **44**, 46.
Rapport, M. M. (1949). *J. biol. Chem.* **180**, 961.
Reid, D. F., Lepkovsky, S., Bonner, D. & Tatum, E. L. (1944). *J. biol. Chem.* **155**, 299.
Sarett, H. P. & Goldsmith, G. A. (1949). *J. biol. Chem.* **177**, 461.
Synge, R. L. M. (1951). *Biochem. J.* **49**, 642.
Synge, R. L. M. & Tiselius, A. (1949). *Acta chem. scand.* **3**, 231.
Tabone, J., Robert, D., Thomassey, S. & Mamounas, N. (1950). *Bull. Soc. Chim. biol., Paris*, **32**, 529.
Williams, R. J. & Kirby, H. (1948). *Science*, **107**, 481.
Wiss, O. (1949). *Helv. chim. Acta*, **32**, 1694.
Wiss, O. & Fuchs, H. (1950). *Experientia*, **6**, 472.
Wiss, O. & Hatz, F. (1949). *Helv. chim. Acta*, **32**, 532.
Wiss, O., Viollier, G. & Waldi, D. (1951). *Helv. physiol. pharmacol. Acta*, **9**, C40.
Yanofsky, C. & Bonner, D. M. (1950). *Proc. nat. Acad. Sci., Wash.*, **36**, 167.

The Virulence-enhancing Factor of Mucins

5. THE DIFFERENT COMPONENTS OF THE 'THIRD FACTOR' INVOLVED IN VIRULENCE ENHANCEMENT

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Previous work (Smith, 1950*a*, *b*, 1951; Smith, Harris-Smith & Stanley, 1951) has shown that the virulence-enhancing action of hog gastric mucin is due to a synergic action between two non-specific factors (a viscous medium and a particulate residue) and a more specific soluble 'third factor'. Partial purification of the latter (Smith, 1951) yielded a heterogeneous product which was predominantly peptide in nature, but which contained 5–10% of carbohydrate residues.

This paper describes further purification of the third factor using a revised assay (Smith *et al.* 1951) in which the two non-specific factors are maintained constant. It has been shown that the carbohydrate moiety is responsible for the activity. Furthermore, this moiety has been separated into at least two active fractions, which have different chemical and biological properties, and which, when combined together, show a synergic effect in the system of the revised assay. This is an important result affecting investigations on the mode of action of mucin.

In the light of this work, various bacterial polysaccharides have been examined for virulence-enhancing activity in the assay for the third factor: they are active and the results are included in this paper, together with a report on their other relevant biological properties.

EXPERIMENTAL AND RESULTS

Methods

Activities are given in 'Virulence-enhancing units' (V.E.U.) (Smith *et al.* 1951) by direct comparison with a standard mucin, with fiducial limits shown in brackets for $P=0{\cdot}95$. The yields and activities of samples obtained from a particular fractionation are quoted for a single experiment. The activities of the various fractions may not be significantly different when taken alone, but become so when coupled with similar results in a number of such fractionations on different batches of the third factor. The inclusion of all the latter results would be superfluous.

Hexosamine, which had been liberated from the various polysaccharides by hydrolysis with HCl at 100° in sealed tubes, was estimated by the method of Elson & Morgan (1933) using the $NaHCO_3$-Na_2CO_3 buffer of Immers & Vasseur (1950) for the acetonylacetone condensation. Replicate assays on different hydrolysates showed the method to have an error of ±5% for most of the compounds used (see also Wolfrom, Weisblat, Karabinos, McNeely & McLean, 1943; Meyer, 1945).

Uronic acid was determined by the carbazole method of Dische (1947), and by the modification due to Tracey (1948) of the classical Lefèvre & Tollens (1907) method. Discrepancies between the two methods for heparin and chondroitin sulphuric acids have been noted by Dische (1947). Replicate assays showed that the error of the Dische method was ±5%, and of the classical method ±3% for the compounds used.

Large osmotic pressures developed during the dialysis of the majority of products, probably due to their highly ionic nature; the cellophan dialysis tubes were therefore protected by calico 'tyres'.

Preparation of crude third factor from fresh hog gastric mucosae

When the full role of a viscous medium and particulate insoluble matter in enhancing virulence was appreciated, and a more accurate biological assay for the third factor was available (Smith *et al.* 1951), the methods already in use (Smith, 1950*b*, 1951) for removing the two non-specific factors from the crude third factor were re-examined. It became apparent that these methods, which were established using an assay (Smith, 1950*a*) now known to be obsolete for determination of the third factor, were essentially sound, and only slight modification was needed to improve yields. The procedure which has been used is as follows:

(1) Autolysis of 200 fresh hog gastric mucosae, precipitation with industrial ethanol, and defatting with trichloroethylene (not dichloroethylene) were as described by Smith (1950*b*).

(2) The viscous constituents were removed at 0° by four aqueous extractions (30, 10, 10 and 10 l.) at pH 4·3–4·5, yielding 800–1000 g. dry matter. This material was assayed for the third factor in two ways. At a concentration of 0·4% (w/v) it was combined with charcoal (0·4%, w/v) and dilute tragacanth mucilage as in the normal procedure for virulence-enhancing samples (Smith *et al.* 1951). At a concentration of 6·5% (w/v) it had an inherent viscosity equal to that used in the normal assay: 0·4% (w/v) charcoal was added and the normal assay procedure was then carried out without the addition of tragacanth mucilage. The former method gave an activity of 0·08 (0·04–0·15) V.E.U./g., and the latter 0·02 (0·01–0·04) V.E.U./g. and a weighted mean of these figures gave 0·05 (0·03–0·07) V.E.U./g.

(3) The crude third factor (130–150 g.) was then extracted from the insoluble residue (120–130 g.) by the method described by Smith (1951) except that the second alkaline

extraction was also made with 1% (w/v) $NaHCO_3$ (48 hr.) instead of with 1% (w/v) Na_2CO_3 (24 hr.). A typical batch had an activity of 0·43 (0·19–0·82) v.e.u./g.

Fractionation between pH's 9 and 5 (Smith, 1951) has been omitted from the process because the above substitution of $NaHCO_3$ for Na_2CO_3 leaves only a small amount of residue at pH 9 in the preparation. This was easily removed, together with the material precipitated by 15% $(NH_4)_2SO_4$, at the next stage of purification: also a relatively large amount of activity remained in the material soluble at pH 5.

Precipitation of extraneous matter with 15% (w/v) ammonium sulphate

Crude third factor (150 g., activity 0·43 (0·19–0·82) v.e.u./g.) was dissolved in water (6%, w/v) at pH 6·9–7·1. Solid $(NH_4)_2SO_4$ was added with stirring to 15% (w/v), the pH was readjusted to 6·9–7·1 and the mixture left at 0–2° for several days. The precipitate was removed by centrifuging at 45 000–50 000 rev./min. in the batch bowl of a Sharples centrifuge which had been refrigerated at 0–5°. The precipitate was dissolved in water and, together with the supernatant, was dialysed at 0° against distilled water until free from $(NH_4)_2SO_4$. To stop bacterial action, a little toluene was added to the material in the bag. Finally, both fractions were freeze-dried. The 15% $(NH_4)_2SO_4$ precipitate, yield 34 g., had activity 0·22 (0·08–0·39) v.e.u./g.; the material soluble in 15% $(NH_4)_2SO_4$ solution, yield 88 g., had activity 0·53 (0·30–0·95) v.e.u./g.

Experiments showed that no further fractionation was possible with higher $(NH_4)_2SO_4$ concentrations: even after saturation with $(NH_4)_2SO_4$ the supernatant contained appreciable activity.

Treatment with trypsin followed by dialysis

Treatment with trypsin resulted in no loss of activity. Subsequent dialysis removed from the active fraction a large proportion of the peptide material (biuret test) and some nucleic acid residues (phosphorus analysis and absorption at 2600 A.).

The material soluble in 15% (w/v) $(NH_4)_2SO_4$ solution (88 g., activity 0·53 (0·30–0·95) v.e.u./g.) was dissolved in water (5%, w/v) containing 1% (v/v) $CHCl_3$ as a preservative; the pH of the mixture was adjusted to 8·0 and the temperature to 37°. Crystalline trypsin (0·125%, calculated on the initial quantity of material soluble in 15% $(NH_4)_2SO_4$ solution) (Armour and Co. Ltd.) was added, and the mixture allowed to stand for 4 hr. at 37°. The large amount of nucleic acid in the mixture provided an effective buffer and the pH dropped very little. The pH was readjusted to 8·0, a further quantity of trypsin (0·125%) was added and the mixture left at 37° for 16 hr.

The action of the trypsin was followed by measuring the liberated α-amino groups using the method of Pope & Stevens (1939). The titration figures showed that the action of the trypsin was almost complete after 1 hr. The fact that maximum action had occurred after 20 hr. was checked by taking a small sample and adding 0·5% trypsin; after a further 1 hr. at 37° no increase in the number of α-amino groups was discernible.

The mixture was then dialysed at 0° against distilled water for several days. The contents of the dialysis bag were freeze-dried. Yield 55 g.; activity 1·0 (0·65–1·5) v.e.u./g. The material passing through the membrane had negligible activity.

Separation of different polysaccharides by fractionating with barium acetate and ethanol, followed by removal of nucleic acid by precipitating with acid

Analysis of barium acetate/ethanol fractions for nucleic acid, peptides and hexosamine; virulence-enhancing activities of selected fractions. The material (2·5 g.), after treatment with trypsin, was dissolved in water (5%, w/v), solid barium acetate was added to 3% (w/v) and the pH adjusted to 6·9–7·1. After standing in the refrigerator overnight, the precipitate that formed was removed by centrifugation at 0°. The supernatant was fractionated by adding various concentrations of ethanol, and, after standing overnight, removing the precipitates at 0°. Each precipitate was dissolved in water (20 ml.) and decomposed with Na_2SO_4 (0·5 g.); $BaSO_4$ was removed by centrifugation and the excess Na_2SO_4 by dialysis, and finally the solution was freeze-dried. The following determinations were made on each fraction: (1) total P; (2) absorption of light of wavelength 2600 A. by the same depth of 0·005% (w/v) aqueous solution; (3) biuret test of Kingsley (1939) on a 0·2% (w/v) aqueous solution; (4) a determination of the hexosamine set free by 16 hr. hydrolysis with 0·5 N-HCl at 100°.

The results, which are shown in Table 1, suggested a procedure in which the following fractions were separated at 0°, decomposed with Na_2SO_4, dialysed and freeze-dried as described above: (1) precipitate on adding 3% (w/v) barium acetate; (2) precipitate on adding ethanol to 10% (v/v); (3) precipitate on adding ethanol to 50% (v/v); (4) supernatant from fraction 3. The yields, analyses and virulence-enhancing activities of these fractions are given in Table 2.

Combination of the barium acetate/ethanol fractionation with removal of nucleic acid by precipitating at pH 2. The fractionation outlined in Table 2, and assays for virulence enhancement on various electrophoretic samples, indicated that neither the nucleic acid nor the peptide material was connected with virulence enhancement. Preliminary experiments on the individual fractions 1, 2 and 3 above showed that the nucleic acid could be removed as a precipitate of low activity by adjusting the reaction of an aqueous solution (5%, w/v) to pH 2, combined with, in the case of fractions 2 and 3, the addition of ethanol to 10 and 20% (v/v) respectively. The active material was then precipitated from the supernatant by adding ethanol to 50% (v/v) and a little sodium acetate. This procedure has now been coupled with the barium acetate/ethanol fractionation. In the process which follows it is imperative that close attention be paid to the temperature of fractionation and centrifuging, and the concentration of constituents, otherwise undue mixing of fractions results.

An aqueous solution (5%, w/v) of trypsin-treated third factor (55 g., activity 1·0 (0·65–1·5) v.e.u./g.) containing barium acetate (3%, w/v) was adjusted to pH 6·9–7·1 and left at 0–2° for several days. The precipitate which formed was removed at 0–2° at 4000 rev./min. in the angle head of a refrigerated centrifuge, washed once with ice-cold 3% (w/v) aqueous barium acetate (100 ml.) and freeze-dried. Fraction *A* (7·5 g.). If the mixture froze at any stage in this process fraction *A* was contaminated with fraction *B*. If this occurred, the mixture was stirred for several hours at room temperature and then cooled again to 0–2°.

Ethanol was added to the supernatant and washings to 10% (v/v) and the mixture was left at 0–2° overnight. The precipitate was removed and washed twice (2 × 100 ml.) as described above with 3% (w/v) barium acetate solution to which ethanol had been added to 10% (v/v). It was then freeze-dried. Fraction *B* (24·9 g.).

The ethanol content of the supernatant and washings was raised to 50% (v/v) and after standing at 0–2°, the precipitate was removed, washed twice (2 × 100 ml.) with 3% (w/v) barium acetate/50% (v/v) ethanol as described above, and freeze-dried. Fraction *C* (19·1 g.). The supernatant was reserved.

Fraction *B* was treated as described for fraction *A* except for two points. After adjusting the pH of the mixture to 1·8–2·0, ethanol was added to 10% (v/v). The final product was precipitated in a rather gelatinous state and was best removed in the batch bowl of the Sharples centrifuge. (Polysaccharide B (5·0 g.); activity 0·74 (0·44–1·3) v.e.u./g.)

Fraction *C* also was treated as described for fraction *A* except for two points. A concentration of 20% (v/v) ethanol was used in the nucleic acid precipitation mixture and 2% (w/v) sodium acetate was added to the 50% (v/v) ethanolic precipitation of the active material (3·6 g.); activity 1·9 (1·3–2·9) v.e.u./g. This material contained hexuronic acid and ester sulphate residues (see below under polysaccharides A and B). Most of these residues could be removed in a precipitate (1·7 g., activity 1·5 (1·2–1·9) v.e.u./g. after decomposing with Na_2SO_4) obtained by adding ethanol to 30% (v/v) to a 5% (w/v) solution at pH 7 containing 3% (w/v) barium acetate. Polysaccharide C was precipitated by raising the ethanol concentration to 50% (v/v). After reaction with Na_2SO_4 and centrifugation to remove $BaSO_4$, polysaccharide C was dialysed against distilled water and freeze-dried. (Yield 1·1 g.; activity 2·1 (1·7–2·6) v.e.u./g.)

Table 1. *Yields and analyses of samples from the barium acetate/ethanol fractionation*

	Fractions precipitated									In supernatant after adding 50% (v/v) ethanol
	By 3% (w/v) barium acetate solution alone	With ethanol added to 3% (w/v) barium acetate solution to give the following % (v/v)								
		2·5	5·0	7·5	10	15	25	35	50	
Yield (%)	10	12	16	8	4	2	0	2	6	10
Absorption of light of wavelength 2600 A. by 0·005% (w/v) solution (1 cm. depth) (%)	47	42	79	88	88	68	55	10	0	7
P (%)	1·8	1·4	4·2	5·1	5·8	3·2	1·0	0·5	0·6	0·25
Biuret test on 0·2% (w/v) solution	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Trace	Strong
Hexosamine liberated by 0·5 N-HCl at 100° for 16 hr. (%)	5·0	22	12·5	4·5	6·3	14·5	15·3	22·0	24·5	7·5

Neg. = negligible.

Table 2. *Yields, analyses and virulence-enhancing activities of selected barium acetate/ethanol fractions*

(Initial trypsin-treated material 0·87 (0·45–1·7) v.e.u./g.)

Fraction no.	Yield (%)	Virulence-enhancing activity (v.e.u./g.)	*P* (%)	Hexosamine set free by 0·5 N-HCl at 100° for 16 hr. (%)	Biuret test
1 (3% Barium acetate, no ethanol)	9	2·7 (1·9–4·0)	1·4	5·5	Negligible
2 (3% Barium acetate, and ethanol added to 10%, v/v)	28	0·84 (0·53–1·3)	4·0	10·3	Negligible
3 (3% Barium acetate and ethanol added to 50%, v/v)	16	1·1 (0·64–1·9)	1·7	12·0	Trace
4 (Supernatant from 3)	8	0·73 (0·49–1·3)	0·4	8·7	Strong reaction

Fraction *A* was suspended in water (6%, w/v), warmed to about 37° and solid Na_2SO_4 (15% of the weight of fraction *A*) was added, and the mixture shaken at room temperature for 0·5 hr. The pH was adjusted to 1·8–2·0 with 12 N-H_2SO_4, and the mixture immediately cooled to 0–2° and left at this temperature overnight. The precipitate of nucleic acid and $BaSO_4$ was removed at 0–2° at 45 000–50 000 rev./min. in the batch bowl of a refrigerated Sharples centrifuge, and reserved. Ethanol was added to the supernatant to 50% (v/v), then 1% (w/v) sodium acetate was added and the mixture was left at −10° overnight. The precipitate was removed at −10 to 0° in the angle head of a refrigerated centrifuge, dissolved in water at pH 7, dialysed for several days against frequent changes of distilled water and freeze-dried. (Polysaccharide A (2·6 g.); activity 4·5 (2·8–7·3)

The nucleic acid precipitates and the 50 % (v/v) ethanolic supernatants from all three fractions were combined with the supernatant containing peptide material from the barium acetate/ethanol fractionation, which had been decomposed with Na_2SO_4. The pH was adjusted to 7 and $BaSO_4$ removed by centrifugation. After dialysis for several days against distilled water it was freeze-dried (30·5 g.). (Activity 0·22 (0·13–0·35) v.e.u./g.)

The chemical nature and biological properties of polysaccharides A, B and C

Table 3 summarizes the important distinguishing properties of these compounds. It includes a determination of anticomplementary activity in a system involving sheep cell haemolysis: our colleague, Dr H. P. Lambert, has shown that such activity is a distinct property of polysaccharide A.

Polysaccharide A has been further purified and obtained essentially homogeneous; it has been identified as a heparin and crystallized as the acid barium salt. The following paper describes its properties in detail, but relevant data are included in Table 3 for comparison with the other polysaccharides.

Polysaccharide B is the sodium salt of a polysaccharide sulphuric ester. The pH (6·0–6·5) of the aqueous solution indicates a mixed acid and neutral salt. It is contaminated slightly with nucleic acid as shown by a phosphorus content of 0·4 % and a slight peak at 2600 A. in the absorption of ultraviolet light in solution. Although it is still impure, the following analysis, and that given in Table 3, indicate that it is predominantly mucoitin monosulphuric acid, or the acid polysaccharide of Meyer, Smyth & Palmer (1937), and Meyer (1938), which was also prepared by Wolfrom *et al.* (1943), and later shown to be a mixture of substances by Wolfrom & Rice (1947). Found: *N*-acetyl (CH_3CO), determined by hydrolysing with 25 % (w/v) *p*-toluenesulphonic acid at 100° overnight and titrating the distilled acetic acid, 5·2; total S (fusion) 5·5; ash 21·7; sulphated ash 22·7; N, 5·2 %. $[\alpha]_D^{20°}$ in water $= -15·5°$ (*c*, 1). Meyer *et al.* (1937) give $[\alpha]_D^{25°} = -20·2$ and $-22·2°$ for two preparations of acid salt and $[\alpha]_D^{24°} = -35·7$ for a neutral preparation. Meyer (1938) gives $[\alpha]_D = -8·1°$ for a neutral sodium calcium salt; Wolfrom *et al.* (1943) give $[\alpha]_D^{25°}$ in water $= -15°$ (*c*, 2) for the neutral sodium salt and $-7·4°$ for the acid sodium salt.

The virulence-enhancing activity of polysaccharide B is low compared with the other polysaccharides, especially with polysaccharide A. It is known that polysaccharide A remains slightly soluble at 0° on the addition of 3 % (w/v) barium acetate at pH 6·9–7·1, and that addition of ethanol to a final concentration of 10 % (v/v) precipitates a large amount of this material. Polysaccharide B, therefore, contains some A, and a rough estimation of the proportion of the latter can be obtained from the figures on the liberation of hexosamine by hydrolysis at 100° with 0·5N- and 3N-hydrochloric acid. Hydrolysis of polysaccharide A at 100° for 16 hr. liberates 7·5 % hexosamine if 0·5N-hydrochloric acid is used, and 25·2 % if 3N-hydrochloric acid is substituted; the latter figure is not increased by further hydrolysis. Hydrolysis of polysaccharide B with 3N-hydrochloric acid for 16 hr. liberates 25·1 % hexosamine, and this is not increased by longer hydrolysis. Fig. 1 shows that the hydrolysis with 0·5N-hydrochloric acid liberates 20 % hexosamine in 8 hr. and this figure is only increased slowly by longer hydrolysis. It is probable that the difference between these values for polysaccharide B is due to the amount of polysaccharide A present. A short calculation shows that the virulence-enhancing activity of polysaccharide B can be largely attributed to the presence of polysaccharide A, and if B has any inherent activity it is very low.

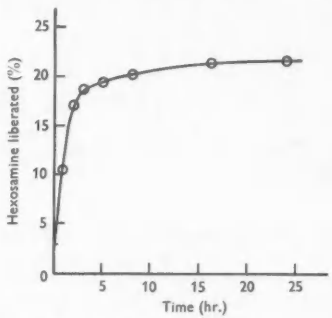

Fig. 1. The liberation of hexosamine from polysaccharide B by 0·5N-HCl at 100°.

Polysaccharide C. Table 3 shows that this compound is different from polysaccharide A. It is still impure (P = 0·2 %, slight positive biuret reaction), but its relatively high activity, coupled with the difference in chemical and biological properties from polysaccharide A, warrant further purification and investigation of this compound or mixture of compounds.

Polysaccharide C must form some complex with protein just as do the sulphated polysaccharides, since it remains insoluble at pH 4·3–4·5, when the crude third factor is washed with water at that pH. Nevertheless, the relatively weak ionic nature of polysaccharide C would tend to make this complex less stable than that of polysaccharides A or B. It is possible, therefore, that the low activity (0·05 (0·03–0·07) v.e.u./g.) of material extracted at pH 4·3–4·5 is due more to polysaccharide C than to A. Investigation of this material as an additional source of polysaccharide C is being carried out.

Table 3. *Virulence-enhancing activities and biological properties of polysaccharides A, B and C, and various bacterial and other polysaccharides: distinguishing analyses of the former, and known chemical nature of the latter*

Polysaccharide	Virulence-enhancing activity of a typical batch (V.E.U./g.)	Anticoagulation activity on fresh ox blood using the technique of Jorpes (1935, 1942): Activity	Anticoagulation activity: Amount added to 100 ml. blood (mg.)	Anticomplementary activity in sheep cell haemolysis: Activity	Concentration in the complement mixture (%, w/v)	S as acid-hydrolysable sulphate (%)	Hexosamine set free by 16 hr. hydrolysis at 100° (%) with: 0·5N-HCl	3N-HCl	Hexuronic acid (%): Dische method	Lefèvre & Tollens method
A	3·9 (2·9–5·1)	Strongly active	0·0125	Strongly active	0·01	11·3	7·5	25·2	34·6	26·6
B	0·69 (0·52–0·90)	Active	1·0	Slightly active	0·025	5·2	20·5	25·1	20·0	27·6
C	1·4 (0·9–2·0)	Inactive	1·0	Inactive	0·025 and 0·075	Approx. 1·0	28·5	28·0	Less than 1·0	Approx. 3
						Known chemical nature of polymer units				
Dextran from *Leuconostoc mesenteroides*	1·6 (1·0–2·7)	Inactive	1·0	Inactive	0·025	Glucose with mainly 1:6 links, branched chain (Evans & Hibbert, 1946)				
Dextran from *Betacoccus arabinosaceus*	1·6 (1·0–2·7)	Inactive	1·0	Inactive	0·025	Glucose with mainly 1:6 links, branched chain (Evans & Hibbert, 1946)				
Dextran from *Leuconostoc dextranicum*	2·0 (1·2–3·3)	Inactive	1·0	Inactive	0·025	Glucose with mainly 1:6 links, straight chain (Evans & Hibbert, 1946)				
Pneumococcus Type I polysaccharide	1·1 (0·6–2·1)	Inactive	1·0	Inactive	0·025	Hexosamine, acetyl residues, D-galacturonic acid (Stacey, 1946)				
Pneumococcus Type V polysaccharide	1·6 (1·0–2·7)	Slightly active	1·0	Slightly active	0·025	Hexosamine, acetyl residues and others (Brown, 1939)				
Polysaccharide from *Azobacter chroöcoccum*	2·4 (1·4–4·3)	Inactive	1·0	Inactive	0·025	D-Glucose, D-galactose, D-glucuronic acid (Lawson & Stacey, 1950)				
Galactogen from trypsin	Negligible activity	Inactive	1·0	Slightly active	0·025	D-Galactose				
Seromucoid	Negligible activity	Inactive	1·0	Inactive	0·025	D-Mannose, D-glucosamine and amino-acids (Stacey, 1946)				

The synergism of a combination of polysaccharides A (or a sample of heparin) and polysaccharide C in the biological assay for the third factor

In consultation with our colleague, Mr S. Peto, experiments were devised to test the synergism of a mixture of polysaccharides A and C in the biological assay for the third factor. In view of the identification of polysaccharide A as a heparin, an authentic sample of heparin was also included in the experiments.

Twenty mice were injected with each concentration in each test and the death rates for all these individual tests were analysed by Mr S. Peto, who has written an addendum to this paper. He found the test for synergism of the combination of polysaccharides A and C to be significant, and the mixture is estimated to be approximately 70% more active than if its constituents acted similarly. This is of the order one would have expected from the recovery of activities mentioned above. In the case of the sample of heparin there is also a strong indication that a combination of it with polysaccharide C is synergic, and it is estimated that the mixture is approximately 50% more active than if its constituents acted similarly.

Table 4. *Synergism of a combination of polysaccharide A (or a sample of heparin) and polysaccharide C in the biological assay for the third factor*

(For the general technique of the biological assay for the third factor see Smith *et al.* (1951). The figures quoted are the average death rates (%) for each concentration in all of four tests, twenty mice being injected with each concentration in each test. The individual tests were analysed by Mr S. Peto, who has written an addendum to this paper. Adequate control experiments rule out any toxicity in the samples injected.)

Injection material	Concn. (%)	Deaths (%)	Concn. (%)	Deaths (%)	Concn. (%)	Deaths (%)
Polysaccharide A	0·05	63	0·025	56	0·0125	50
Polysaccharide C	0·1	77	0·05	65	0·025	50
Polysaccharide A 2 parts Polysaccharide C 3 parts	0·07	89	0·035	68	0·0175	54
Heparin (Boots Pure Drug Co. Ltd.)	0·05	75	0·025	57	0·0125	42
Heparin (Boots Pure Drug Co. Ltd.) 2 parts Polysaccharide C 3 parts	0·07	93	0·035	54	0·0175	60

This series of experiments was prompted by two facts. First, the wide difference in chemical and biological properties of the two polysaccharides suggested that they might impede different host defence mechanisms, and some synergic effect would be expected. Secondly, the total yield of activity (based on the most probable figures) of all the fractions recovered in the barium acetate/ethanol fractionation was low (50–70%); this would not be surprising for one fractionation, in view of the fiducial limits of the virulence-enhancing activities, but all such fractionations yielded similar figures. There was a loss of material through dialysis membranes when the complexes of protein with nucleic acid and polysaccharide were split, but we have no evidence of loss of activity through these membranes.

Polysaccharide A (activity 3·9 (2·9–5·1) v.e.u./g.) and a sample of heparin (Boots Pure Drug Co. Ltd., activity 3·2 (2·1–4·8) v.e.u./g.) were tested in the biological assay for the third factor at concentrations of 0·05, 0·025 and 0·0125% (w/v) and a sample of polysaccharide C (activity 2·1 (1·7–2·6) v.e.u./g.) at concentrations of 0·1, 0·05 and 0·025% (w/v). In the same test, mixtures of 2 parts of polysaccharide A or heparin and 3 parts of polysaccharide C were injected at concentrations of 0·07, 0·035 and 0·0175% (w/v), which should give approximately the same death rates as the individual samples if no synergism occurred. This test was repeated four times with the same batch of polysaccharide C.

Table 4 summarizes these experiments. For brevity, the average death rates (%) for each concentration of the materials used in all four tests is quoted instead of the individual values.

The virulence-enhancing activities of various bacterial polysaccharides: their chemical nature and biological properties

The fact that the activity of the third factor lies in the polysaccharide moiety prompted an investigation of several bacterial polysaccharides for virulence enhancement, anticoagulation and anticomplementary activity. Prof. M. Stacey and Dr W. G. Overend of the University of Birmingham kindly supplied us with a number of such polysaccharides. Two of animal origin—a galactogen from trypsin and seromucoid—have also been included in the investigation. The virulence-enhancing activity was determined at concentrations 0·1, 0·05, 0·025 and 0·0125% (w/v) in the normal assay for the third factor (Smith *et al.* 1951), making certain that the inherent viscosity of some of the preparations did not affect unduly the 'standard' viscosity of the solutions used in the assay.

The results of this investigation, together with a brief outline of the known chemical nature of these compounds are included in Table 3 for comparison

with polysaccharides A, B and C. All the bacterial polysaccharides were active in enhancing virulence, yet the two of animal origin had negligible activity at the concentrations used.

DISCUSSION

The fourth paper in this series (Smith *et al.* 1951) made it clear that the virulence-enhancing action of hog gastric mucin was due to a synergic combination of a viscous medium, a particulate residue and a soluble third factor. In the present paper the activity of the crude third factor (Smith, 1951) has been shown to reside in the carbohydrate moiety: furthermore, this moiety has been separated into at least three different parts, two of which—polysaccharides A and C—are involved in the virulence enhancement.

Polysaccharide A has been obtained essentially homogeneous and identified as a heparin (see following paper). Polysaccharide B is impure, but is predominantly mucoitin monosulphuric ester or the 'acid polysaccharide' of Meyer *et al.* (1937). Its laevorotation, ester sulphate analysis and the presence of acetyl residues show that it is different from polysaccharide A and heparin. It has a low activity which can be largely, if not totally, attributed to the presence of polysaccharide A. Purification of this compound with regard to its virulence-enhancing activity, therefore, has been discontinued. Polysaccharide C is as yet impure, but it is clearly different from polysaccharide A. It has a relatively high activity and further fractionation of this compound or mixture of compounds is being continued.

Coupled with the difference in the chemical and especially the ionic nature of polysaccharides A and C is a marked difference in biological properties. Polysaccharide A is a powerful anticoagulant for blood and has a strong anticomplementary effect in a system involving sheep cell haemolysis; polysaccharide C is devoid of any such activity. It is quite possible, therefore, that the two polysaccharides may have different modes of action in helping to enhance virulence; this view is strongly supported by the synergic effect of a combination of the two in the assay for the third factor involved in virulence enhancement. Investigations into the mode of action of mucin in enhancing virulence must take account of this possibility, in addition to the effects of the particulate matter and of a viscous medium. The virulence-enhancing action of hog gastric mucin is an excellent example of biological activity being due to a system of interacting factors, rather than to one factor alone. The relative importance of the various factors operating might well vary with the nature of the organism establishing the infection and the particular host defence mechanisms called into play in combating that infection.

Polysaccharides of non-pathogenic bacteria (levans from *Aerobacter levanicum* and *Bacillus subtilis*, dextran from *Leuconostoc mesenteroides* cellulose from *Acetobacter xylinum*) and of pathogenic bacteria (*Escherichia coli*, *Salmonella typhi*, *Salmonella enteritidis*, *Klebsiella capsulata*, *Pneumococci* Types I and II) have been shown to have virulence-enhancing activity at concentrations of 0·2–2·0% (Olitzki, 1948; Olitzki, Shelubsky & Hestrin, 1946; Felton & Bailey, 1926; Ward, 1930). When examined in the biological assay for the third factor, in which samples are injected in the presence of a fixed amount of particulate residue and in a medium of constant viscosity (Smith *et al.* 1951), various bacterial polysaccharides proved active at low concentrations (0·1% (w/v) and lower). The interesting fact is that the activities of these compounds are of the same order as polysaccharide C, which they also resemble in containing no large amounts of highly ionic sulphate residues, and in having no anticoagulatory or anticomplementary action. It is relevant to point out that the virulence-enhancing activity or 'aggressin effect' of Types I, II and III pneumococcus polysaccharides has been attributed to some extent to their antiopsonic and antiphagocytic action (for discussion see Wilson & Miles, 1946).

Although the above compounds have been designated polysaccharides, Stacey (1946) believes that all bacterial polysaccharides retain a small amount of amino-acid residues which are important as regards serology and antigenicity. Mucopolysaccharides is perhaps a better name for these compounds, and for polysaccharide C; the latter still contains amino-acid residues which may have an important bearing on its activity.

SUMMARY

1. The virulence-enhancing activity of the third factor of hog gastric mucin resides in at least two predominantly polysaccharide fractions.

2. These fractions differ in chemical nature and in some biological properties. The first, which has been purified and identified (see following paper), contains highly ionic sulphate residues and has an intense anticoagulatory and anticomplementary activity; the second fraction, which is still impure, is devoid of such residues and activities. When combined together in the biological assay for the third factor the two fractions have a synergic effect.

3. Various bacterial polysaccharides have virulence-enhancing activities and other biological properties similar to the second of these fractions.

Our thanks are due to Dr H. P. Lambert for carrying out determinations of anticomplementary action, and to Mr R. Strange for micro-analyses.

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REFERENCES

Brown, R. (1939). *J. Immunol.* **37**, 445.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Evans, T. H. & Hibbert, H. (1946). *Advances in Carbohydrate Chemistry*, vol. II, p. 204. New York: Academic Press.
Felton, L. D. & Bailey, G. H. (1926). *J. infect. Dis.* **38**, 131.
Immers, J. & Vasseur, E. (1950). *Nature, Lond.*, **165**, 898.
Jorpes, E. (1935). *Biochem. J.* **29**, 1817.
Jorpes, E. (1942). *Biochem. J.* **36**, 203.
Kingsley, G. R. (1939). *J. biol. Chem.* **131**, 197.
Lawson, G. J. & Stacey, M. (1950). *Biochem. J.* **46**, xiii.
Lefèvre, K. U. & Tollens, B. (1907). *Ber. dtsch. chem. Ges.* **40**, 4513.
Meyer, K. (1938). *Cold Spr. Harb. Sym. quant. Biol.* **6**, 91.
Meyer, K. (1945). *Advances in Protein Chemistry*, vol. II, p. 256. New York: Academic Press Inc.
Meyer, K., Smyth, E. M. & Palmer, J. W. (1937). *J. biol. Chem.* **119**, 73.
Olitzki, L. (1948). *Bact. Rev.* **12**, 149.
Olitzki, L., Shelubsky, M. & Hestrin, S. (1946). *Proc. Soc. exp. Biol., N.Y.*, **63**, 491.
Pope, C. G. & Stevens, M. F. (1939). *Biochem. J.* **33**, 1070.
Smith, H. (1950*a*). *Biochem. J.* **46**, 352.
Smith, H. (1950*b*). *Biochem. J.* **46**, 356.
Smith, H. (1951). *Biochem. J.* **48**, 441.
Smith, H., Harris-Smith, P. W & Stanley, J. L. (1951). *Biochem. J.* **50**, 211.
Stacey, M. (1946). *Advances in Carbohydrate Chemistry*, vol. II, p. 162. New York: Academic Press Inc.
Tracey, M. V. (1948). *Biochem. J.* **43**, 185.
Ward, H. K. (1930). *J. exp. Med.* **51**, 685.
Wilson, G. S. & Miles, A. A. (1946). *Topley and Wilson's Principles of Bacteriology and Immunity*, vol. II, p. 1071. London: Edward Arnold.
Wolfrom, M. L. & Rice, F. A. H. (1947). *J. Amer. chem. Soc.* **69**, 2918.
Wolfrom, M. L., Weisblat, D. I., Karabinos, J. V., McNeely, W. H. & McLean, J. (1943). *J. Amer. chem. Soc.* **65**, 2077.

ADDENDUM

Statistical Treatment of Data Regarding the Synergism of Mixtures of Polysaccharides A and C

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METHOD

The general details of the tests involving polysaccharide A (A), a sample of heparin (H), polysaccharide C (C) and mixtures of the latter with the other two (A/C and H/C) are given above.

Assuming similar synergic action five parallel probit-log dose regression lines ($A, H, C, A/H, H/C$) have been fitted for each of the four replicate experiments. The mean probit difference ($\Delta \pm$ its standard deviation) between the observed probit-dosage relationship of the mixture and that predicted according to the hypothesis of similar action has been calculated as described by Finney (1947) and used as a measure of the synergism. In addition, the logarithm ($M \pm$ its standard deviation) of the ratio of the observed potency of the mixture to that predicted on the above-mentioned hypothesis was calculated.

These results (Δ's and M's) for the four individual experiments have been combined using three different methods:

(1) Taking weighted means with reciprocal variances as weights and the reciprocal of the sum of the weights as the variance of the weighted mean.

(2) Constructing a new set of regression lines with $\Sigma Snwx^2$, $\Sigma Snwxy$, $\Sigma Snwy^2$, etc.

(3) By adding corresponding mortalities and

Table 1. *Mean probit differences* (Δ) *and the logarithms* (M) *of the ratio of observed potency of the mixture to that predicted on the hypothesis of similar action of mixtures* (A/C, H/C) *of polysaccharide A, a sample of heparin* (H) *and polysaccharide C*

Method of calculation	$\Delta\ A/C$	$M\ A/C$	$\Delta\ H/C$	$M\ H/C$
1	$0{\cdot}242 \pm 0{\cdot}134$	$0{\cdot}244 \pm 0{\cdot}108$	$0{\cdot}234 \pm 0{\cdot}136$	$0{\cdot}196 \pm 0{\cdot}102$
2	$0{\cdot}311 \pm 0{\cdot}106$	$0{\cdot}236 \pm 0{\cdot}084$	$0{\cdot}242 \pm 0{\cdot}106$	$0{\cdot}184 \pm 0{\cdot}083$
3	$0{\cdot}305 \pm 0{\cdot}106$	$0{\cdot}261 \pm 0{\cdot}099$	$0{\cdot}245 \pm 0{\cdot}253$	$0{\cdot}168 \pm 0{\cdot}202$

fitting two groups of three probit-dosage regression lines, namely, *A*, *C*, *A*/*C* and *H*, *C*, *H*/*C*.

Table 1 summarizes these combined results.

DISCUSSION

The assumption of parallelism was not contradicted in three of the individual comparisons, but the fourth, which was somewhat erratic, showed significant departure from parallelism. On combining the results as described in (2) and (3) above only (3) *A*/*C* showed a deviation from parallelism. In all four experiments there was a tendency for the regression lines of the mixtures to be steeper than those of their constituents. The inflated variances of (3) *H*/*C* are due to a large heterogeneity factor.

The significance of the synergism of a mixture of polysaccharides A and C is undoubtedly shown by these tests. This mixture is estimated to be approximately 70% more active than if its constituents acted similarly: the corresponding figure for the mixture of the sample of heparin and polysaccharide C is approximately 50%.

REFERENCE

Finney, D. J. (1947). *Probit Analysis.* Cambridge University Press.

The Virulence-enhancing Factor of Mucins

6. IDENTIFICATION OF A HEPARIN AS THE MAIN COMPONENT OF THE THIRD FACTOR INVOLVED IN VIRULENCE ENHANCEMENT

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The third factor involved in the virulence-enhancing action of hog gastric mucin has been shown to be of predominantly carbohydrate nature, and has been split into two quite different active parts named polysaccharides A and C, which form a synergic combination (Smith, Gallop & Stanley, 1952). This paper describes the purification of polysaccharide A, the evidence that it is essentially homogeneous, its identification as a heparin, and its crystallization as an acid barium salt.

EXPERIMENTAL

The same remarks made in the previous paper (Smith *et al.* 1952) regarding the activities of virulence-enhancing samples, and analyses for hexosamine and hexuronic acid, are applicable here.

Purification of polysaccharide A

Polysaccharide A (4·3 g.), prepared as described by Smith *et al.* (1952), was dissolved in water (86 ml.). Solid barium acetate (3%, w/v) was added, the pH was adjusted to 6·9–7·1 and the mixture was left at 0–2° for several days. The precipitate was collected in a refrigerated centrifuge at 0–2° and washed twice (2 × 9 ml.) with ice-cold barium acetate solution (3%, w/v). The neutral Ba salt was dissolved in water (100 ml.) at pH 7·0 and Na_2SO_4 (1 g.) was added. The $BaSO_4$ was removed by centrifugation and the solution was dialysed against distilled water at 0°, in a cellophan tube strengthened by an outer 'tyre' of calico, until free from sulphate. A trace of colloidal $BaSO_4$ was removed by filtering through well washed 'Celite' filter aid, and the solution was freeze-dried; yield 3·2 g., activity 3·9 (2·9–5·1) virulence-enhancing units (V.E.U.)/g. The pH of an aqueous solution was approximately 6, indicating that a mixture of acid and neutral Na salt (see below) was produced owing to a slight loss of sodium ions during dialysis.

When the polysaccharide had been identified as a heparin, it was found that the acid Ba salt could be crystallized directly from the neutral Ba salt, prepared as described above. Neutral Ba salt (50 mg.) was dissolved in warm water (2 ml.) and a trace of insoluble matter was removed by centrifugation. The solution was heated to 65° and glacial acetic acid (0·3 ml.) added; the mixture was cooled slowly to room temperature and then in a refrigerator. The sheaves of crystals (30 mg.) were removed by centrifugation, washed with 50% acetic acid, glacial acetic acid, absolute ethanol and ether, and dried in a vacuum desiccator over P_2O_5.

Tests for homogeneity

Electrophoresis. An investigation carried out by our colleague, Dr B. R. Record, is described in the addendum to this paper; it showed polysaccharide A to be electrophoretically homogeneous at pH's 8·0 and 4·5.

Analyses carried out by us on the material migrating as the single peak, and on that in the stationary boundary, are given in Table 1; these analyses are proof that the latter was an artifact.

Ultracentrifugal analysis. Polysaccharide A was examined in a Spinco ultracentrifuge by Dr J. M. Creeth of the Courtauld Institute of Biochemistry, Middlesex Hospital, London, who writes in the addendum to this paper.

A 1% (w/v) solution in phosphate buffer at pH 8·0 and in acetate buffer at pH 4·5 gave sedimentation diagrams which were characteristic of a single homogeneous substance.

Fractional solubility test. The modification by Aminoff, Morgan & Watkins (1950) of the classical equilibrium method of solubility test was used because of the extreme solubility in water of polysaccharide A.

The chemistry of polysaccharide A, its identification as a heparin

The chemistry of polysaccharide A outlined below leaves no doubt that it is a heparin. Heparins are polymeric mucoitin sulphuric acids in which the number of sulphate groups can vary considerably (Jorpes & Gardell, 1948); details of the chemistry are given by Reinert & Winterstein (1939), Charles & Todd (1940), Wolfrom, Weisblat, Karabinos, McNeely & McLean (1943), Wolfrom & Rice (1946, 1947), Jorpes, Boström & Mutt (1950), Wolfrom, Montgomery, Karabinos & Rathger (1950) and Meyer & Schwartz (1950). Samples of heparin used by these workers were, in the main, obtained from liver and lung tissue; the analyses indicated the presence of an average of two to three sulphate groups for each hexosamine-uronic acid unit in the polymer chain.

Table 1. *Analysis and virulence-enhancing activities of polysaccharide A and preparations obtained on its electrophoresis*

(A 2% (w/v) solution of polysaccharide A was used for electrophoresis and the samples (approx. 3 ml.) collected from the apparatus by Dr B. R. Record.)

Preparation	Electrophoretic fraction (%, w/v, from refractive index difference)	Wt. of material recovered* (mg.)	Analyses†			
			Hexuronic acid (Dische method)	After 16 hr. hydrolysis with 3N-HCl: Hexosamine (%)	After 16 hr. hydrolysis with 3N-HCl: SO_4	Virulence enhancing activity (v.e.u./g.)
Polysaccharide A	·	·	34·6	25·2	+	3·9 (2·9–5·1)
Acetate buffer, pH 4·5:						
Migrating component	1·0	27	35·2	24·1	+	4·1 (2·0–7·6)
Stationary boundary	0·4	Less than 1	Nil	Nil	Nil	·‡
Phosphate buffer, pH 8·0:						
Migrating component	0·9	25	35·2	25·0	+	4·1 (2·0–7·6)
Stationary boundary	0·4	Less than 1	Nil	Nil	Nil	·‡

* After dialysis and freeze-drying. For the stationary boundary only half (1·5 ml.) dialysed.

† For migrating fractions, on material recovered after dialysis; for stationary boundary, on the solution from electrophoresis.

‡ There was insufficient material for an animal test in this experiment, but in a similar experiment, the material from the stationary boundary had negligible activity.

Table 2. *Fractional solubility test on polysaccharide A*

Analysis	Fraction 1	2	3	4	5
$[\alpha]_D^{20°} \pm 10°$	+50	+50	+52·5	+55	+52·5
Hexosamine (%)	25·3	24·0	23·8	23·6	25·2
Hexuronic acid (%) (Dische, 1947)	34·6	36·0	34·9	36·4	37·0
S (as acid-hydrolysable sulphate %)	10·6	10·8	11·0	11·2	11·2

A small quantity (150 mg.) of polysaccharide A was shaken vigorously with successive quantities (8–10 ml.) of 50% (v/v) aqueous ethanol at room temperature for 0·5 hr. After each addition of aqueous ethanol and subsequent shaking, the suspension was left at 0–2° overnight and centrifuged at this temperature. The material (approx. 20 mg.) was recovered from each supernatant by drying over P_2O_5 at 0·1 mm. Hg and was dissolved to give solutions of known concentration. These solutions were analysed for optical rotation, hexosamine liberated by 16 hr. hydrolysis at 100° with 3N-HCl, hexuronic acid by the Dische method (1947), and S as sulphate hydrolysable by 6N-HCl in 16 hr. at 100°. The results are given in Table 2, and bearing in mind the limits of the assays on such small quantities of material, no heterogeneity is indicated by these figures.

Elementary analysis, hexosamine and hexuronic acid analysis and optical rotation. Table 3 shows the analytical figures for polysaccharide A together with those published by Reinert & Winterstein (1939) and Wolfrom *et al.* (1943)

Table 3. *Analytical figures for polysaccharide A and the neutral sodium salt of heparin*

		Heparin				
		Reinert & Winterstein (1939).	Wolfrom *et al.* (1943)		Calc. for mucoitin disulphuric acid	
	Polysaccharide A. Mixture of neutral and acid Na salt	Neutral sodium salt from crystalline barium salt	'Roche' heparin neutral sodium salt	Neutral sodium salt from crystalline barium salt	Neutral salt, $C_{12}H_{16}O_{16}NS_2Na_3$	Acid salt, $C_{12}H_{17}O_{16}NS_2Na_3$
C (%)	25·3	23·4	.	.	25·6	26·6
H (%)	3·7	3·5	.	.	2·8	3·2
N (%)	2·5 (Dumas) 2·7 (Kjeldahl)	2·6	2·6	.	2·5	2·6
S (%)	11·7	11·9	11·7	.	11·3	11·8
Na (from sulphated ash, %)	10·5	11·6	12·0	12·4	12·2	8·5
P (%)	Nil	Nil	0·1	.	Nil	Nil
Hexosamine (%)	.	.	.	.	31·7	33·0
(1) Elson & Morgan (1933) after 3 N- or 4 N-HCl hydrolysis	25·2	.	24·7	23·8	.	.
(2) Van Slyke after 3 N-HCl hydrolysis (100°, 24 hr.)	30·0	.	.	.	.	.
Hexuronic acid (%)	.	.	.	.	34·4	35·8
(*a*) Lefèvre & Tollens method	26·6	.	24·5	.	.	.
(*b*) Dische method (1947)	34·6	.	.	.	.	.
$[\alpha]_D^{20-25°}$ in water	+53° (*c*, 1) (±2)	+45° (*c*, 1 corrected for 6·9% water)	+42° (*c*, 2)	+55° (*c*, 2)	.	.

for the neutral Na salts of different samples of heparin, and those calculated for an average polymer unit composed of a mucoitin disulphuric acid. Hydrolysis with 3 N-HCl at 100° was needed to liberate the maximum quantity of hexosamine; only 7·5 % was set free by 16 hr. hydrolysis with 0·5 N-HCl: some resistance to acid hydrolysis has been noted for heparin (Jorpes, 1946).

The sample contained no inorganic sulphate. After hydrolysis with 3 N-HCl for 6 hr., sulphate was recovered equivalent to 11·3 % S: this shows that all the S was present as ester sulphate.

Amino N. (*a*) *Van Slyke method.* After hydrolysis with 3 N-HCl at 100° for 24 hr. polysaccharide A gave 2·35 % amino N: glucosamine hydrochloride gave a recovery of 99·4 % after the same hydrolysis. With 6 N-HCl at 100° for 24 hr. the corresponding figures were: polysaccharide A, 2·31 %, glucosamine hydrochloride, 87·8 % recovery.

On applying the Van Slyke method to polysaccharide A without previous hydrolysis, the amino N figure increased with the time of reaction with HNO_2 at 25° (2 min. 0·47 %, 30 min. 1·03 %, 60 min. 1·34 %, 90 min. 1·97 %). Extrapolation to zero time gave a strong indication that the hexosamine amino group is fully substituted. The same result has been reported for heparin (Wolfrom *et al.* 1943).

(*b*) *Ninhydrin method.* No α-amino N was detectable in polysaccharide A by the ninhydrin method after 24 hr. hydrolysis with 6 N- and 3 N-HCl. This indicates the absence of amino-acid residues, and it is confirmed by evidence from paper chromatography and absorption of ultraviolet light (see below).

Paper chromatography. (*a*) D-*Glucosamine.* 24 hr. (100°) hydrolysates of polysaccharide A with 3 N- and 6 N-HCl were examined on one-dimensional paper chromatograms using a mixture of collidine and lutidine as solvent. On developing with alkaline acetonylacetone followed by *p*-dimethylaminobenzaldehyde (Partridge, 1948), the presence of D-glucosamine and the absence of chondrosamine was indicated.

(*b*) *Absence of amino-acids.* The same hydrolysates were examined on a two-dimensional paper chromatogram using phenol and collidine as solvents and ninhydrin as developer. No amino-acid spot was visible.

(*c*) *Absence of various sugars.* 1, 5 and 24 hr. hydrolysates (100°) of polysaccharide A with 0·1 N-, 0·5 N- and 3 N-HCl were examined on one-dimensional paper chromatograms, using butanol and a mixture of collidine and lutidine as solvents and aniline phthalate as developer. Glucose, ribose, galactose, fructose, mannose, xylose and galacturonic acid were also placed on the same paper. No sugar spot, but only pink smears, were apparent in the hydrolysates. Furfural, which is formed readily from D-glucuronic acid, gives a pink colour with the developer, but is too volatile to give a definite spot on the paper (see also Partridge, 1950).

Absence of N-*acetyl groups.* After much controversy, it is now generally agreed that heparins do not contain any appreciable quantity of *N*-acetyl groups (Wolfrom *et al.* 1943; Jorpes *et al.* 1950). On hydrolysis with 25 % (w/v) *p*-toluenesulphonic acid, both under reflux for 2 hr. and in a sealed tube at 100° for 16 hr., the maximum amount of distillable acid from several different quantities of polysaccharide A was equivalent to less than 0·5 % *N*-acetyl (CH_3CO—). The same method gave consistent results for the appreciable *N*-acetyl groups in polysaccharide B (see Smith *et al.* 1952), and in a sample of crude blood group A substance.

Emission spectroscopy for metals. This was carried out by Mr L. C. Thomas of the Chemical Defence Experimental Station, Porton, Wilts. A solution of polysaccharide A (not the ash) was sprayed into a flame. The emission spectrum produced indicated the presence of 9·4 % Na, 0·2 % Ca and traces of Al, Si, Mg, Cu, Ag and Fe.

Absorption of ultraviolet light. The ultraviolet light absorption of polysaccharide A was examined in a Unicam quartz spectrophotometer in a cell 1 cm. thick. A 0·05 % (w/v) solution of polysaccharide A in water at pH 7 gave an absorption curve which is shown in Fig. 1. There is no significant increase in absorption at 2600 and 2800 A. Nucleic acids and aromatic amino-acids, respectively, absorb ultraviolet light at these wavelengths.

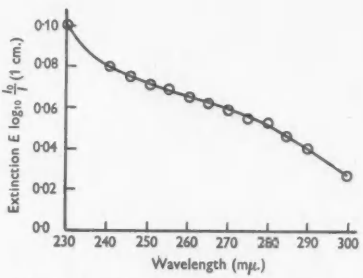


Fig. 1. The ultraviolet absorption spectrum of polysaccharide A (0·05 % (w/v) in water, pH 7).

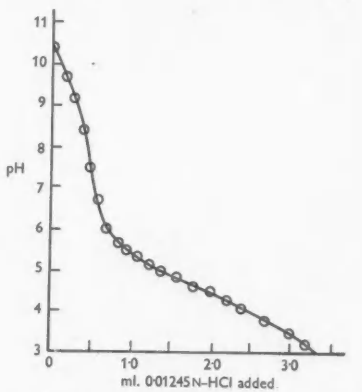


Fig. 2. Potentiometric titration of polysaccharide A (20 mg.).

Potentiometric titration. Polysaccharide A (20 mg.) was dissolved in water (2·5 ml.); one drop of 0·5 N-NaOH was added and the pH rose from 6·1 to 10·4. This solution was then titrated against 0·01245 N-HCl, the pH being measured by a glass electrode. The curve (Fig. 2) for this titration shows that the uronic acid carboxyl group is free, as is that in heparin (Wolfrom *et al.* 1943; Jorpes, 1946). The amount of acid (2·7 ml.) required to carry the titration from pH 6·5 to 3·0 indicates an approximate equivalent weight of 595. Di- and tri-sodium salts of mucoitin disulphuric acid would have equivalent weights of 541 and 563 respectively.

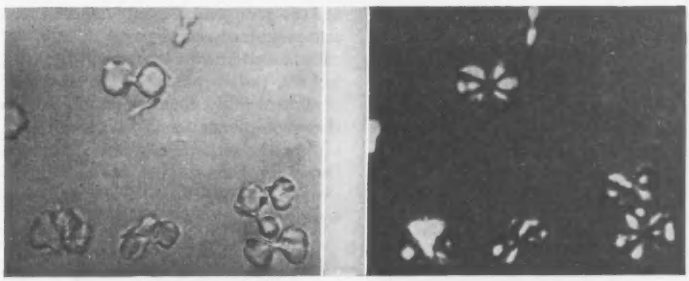
Isolation of D-*glucosamine hydrochloride.* The Ba salt of polysaccharide A (0·5 g.) was refluxed for 16 hr. with 3N-HCl (50 ml.). Approximately the correct quantity of extra Ba needed to precipitate the excess sulphate was added as $BaCl_2$. After removing the $BaSO_4$ by centrifugation, the liquid was concentrated to a syrup under reduced pressure to remove excess HCl. Water (50 ml.) was added, the solution was freed from barium and sulphate ions and evaporated to a syrup. After standing in a refrigerator overnight crystals formed, the quantity being increased by cautiously adding acetone. These crystals (90 mg., $[\alpha]_D^{25°}$ in water = +66·2° (*c*, 0·4), 96·4 % hexosamine hydrochloride by the method of Elson & Morgan, 1933) were recrystallized from a mixture of water, methanol and acetone. Found: N, 6·5. Calc. for $C_6H_{13}O_5N$, HCl: N, 6·5 %; $[\alpha]_D^{25°}$ in water = +71°±2° (*c*, 1), after 24 hr.; the generally accepted value for glucosamine hydrochloride is $[\alpha]_D^{25°}$ = +72·5°. As a final check on the identity, an X-ray powder photograph gave a diffraction diagram which was the same as that for an authentic specimen of D-glucosamine hydrochloride and not that for an authentic specimen of chondrosamine hydrochloride. We are indebted to Prof. A. R. Todd, F.R.S., and Mr A. R. Gilson of the Chemical Laboratory, Cambridge, for these X-ray powder photographs, and to Dr W. T. J. Morgan, F.R.S., for the gift of the chondrosamine hydrochloride.

Molecular weight. Dr B. R. Record and Dr J. M. Creeth, in their addendum to this paper, give the approximate molecular weight of polysaccharide A as 16 500 from a combination of ultracentrifuge and diffusion data.

Crystallization of polysaccharide A as an acid barium salt

Polysaccharide A (0·5 g.) was dissolved in water (20 ml.) and 5 % (w/v) barium acetate (7 ml.) was added. After standing 4 days at room temperature, a trace of insoluble matter was removed by centrifugation. To the supernatant at 65° glacial acetic acid (5 ml.) at the same temperature was added; the mixture was allowed to cool slowly to room temperature and then in a refrigerator. The sheaves of crystals (0·35 g.) were collected by centrifugation, washed with 50 % acetic acid, glacial acetic acid, absolute ethanol and ether, and dried in a vacuum desiccator over P_2O_5. Fig. 3 shows photomicrographs of the crystals in ordinary and in polarized light. We are indebted to our colleague Mr E. O. Powell for these photographs.

Found on material dried at 60° over P_2O_5 at a pressure of 0·1 mm. Hg, C, 22·3; H, 3·4; N, 2·4; S, 9·9; Ba, 22·3; ash (sulphated), 36·7 %; hexosamine liberated by 16 hr. hydrolysis with 3N-HCl, 22·8 %; hexuronic acid by the method of Tollens & Lefèvre, 23·3 %, by the method of Dische (1947) 29·9 %. Calc. for the acid Ba salt of a polymer with an average unit of mucoitin disulphuric acid, i.e. $C_{12}H_{17}O_{16}NS_2Ba$, C, 22·8; H, 2·7; N, 2·2; S, 10·1; Ba, 21·7; ash (sulphated), 36·8; hexosamine, 28·2; hexuronic acid, 30·4 %.



(*a*) (*b*)

Fig. 3. Crystals of the acid barium salt of polysaccharide A in ordinary (*a*) and polarized (*b*) light. Magnification, × 500.

The acid Ba salt of polysaccharide A had $[\alpha]_D^{22°}$ in water = +47° (±2°) (*c*, 1). Wolfrom *et al.* (1943) give $[\alpha]_D^{25°}$ in water = +44° and +47·5° (*c*, 2) for two separate samples of the acid Ba salt of heparins.

After decomposing with sodium sulphate and removing the $BaSO_4$ by centrifugation, the crystals of the acid Ba salt of polysaccharide A had a virulence-enhancing activity of 3·5 (2·2–5·3) V.E.U./g.

The anticoagulation activity of polysaccharide A and the virulence-enhancing activities of authentic samples of heparin

Table 4 shows the anticoagulation activity of polysaccharide A compared with that of three different, highly active, samples of heparin from Boots Pure Drug Co. Ltd. (102 i.u./mg.), Evans, Lescher and Webb Ltd., (93 i.u./mg.) and Roche Products Ltd. (120 i.u./mg.). Fresh ox blood (3·75 ml.) was mixed with solutions (0·3 ml.) of different strengths of the samples under test and the times for complete coagulation were noted, using the technique described by Jorpes (1935, 1942). Polysaccharide A has a slightly greater activity than that of the three authentic heparin samples.

Corresponding virulence-enhancing activities are: polysaccharide A 3·9 (2·9–5·1), Boots heparin 3·2 (2·1–4·8), Evans, Lescher & Webb heparin 2·2 (1·4–3·3), Roche heparin 3·5 (2·3–5·1) V.E.U./g., determined by the assay used for the third factor

involved in virulence enhancement (Smith, Harris-Smith & Stanley, 1951). Adequate controls ruled out any possibility of the toxicity of these samples being a factor in the virulence enhancement at the concentration used for the test.

In addition to these commercial samples of heparin, Dr Bergel of Roche Products Ltd. kindly supplied us with a purified sample of sodium heparinate with the following analytical figures: S, 13·3, ash 40·2 %, $[\alpha]_D = +42·5°$ for the anhydrous material. The material, which contained 8·1 % of water, had an anticoagulation activity 130 i.u./mg. This sample had a virulence-enhancing activity of 3·5 (2·3–5·4) v.e.u./g.

In addition to the similar virulence-enhancing activities and anticoagulation activities of polysaccharide A and heparin, our colleague Dr H. P. Lambert had found that polysaccharide A inhibits sheep cell haemolysis, and that this effect is due to an anticomplementary action. Heparin has already been shown to be anticomplementary (Ecker & Cross, 1929).

sulphuric acid, and had a small positive optical rotation (+2°) and an appreciable acetyl content (6·4 %). It was tested for heparin activity, but it was reported that it contained only 1 % of the activity of the crystalline barium salt of heparin. No criteria of purity were given for any of these preparations, and they were undoubtedly mixtures of compounds. We would point out here that our crude hog gastric mucin and commercial samples are made from the whole mucosae. The excreted mucus itself, however, has appreciable virulence-enhancing activity (Smith 1951*b*), and tests on the 'peptic' extract (see Smith, 1951*b*) of this mucus showed it to have appreciable anticoagulant activity.

It is well known that, owing to their highly ionic nature, polysaccharide sulphates resemble nucleic acid in readily forming complexes with proteins, these complexes being sparingly soluble at pH values on the acid side (Meyer, 1938). It is not surprising, therefore, that impure samples of the third factor (Smith, 1950*b*, 1951*a*) contained much protein and were sparingly soluble below pH 5. This strong

Table 4. *Anticoagulation test on polysaccharide A and three different heparin samples*

(Technique using fresh ox blood as Jorpes, 1935, 1942.)

Strength of anticoagulant solution (%, w/v)	Anticoagulant (mg./100 ml. blood)	Time for complete coagulation (hr.)							
		Polysaccharide A		Boots heparin		Evans heparin		Roche heparin	
		Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
0·0156	1·25	No clot	96	84	84	24	36	48	36
0·0125	1·0	No clot	48	24	36	18	36	48	36
0·00625	0·5	4	5	5	4	2·5	5	9	4
0·00313	0·25	2	2	1·25	1·25	1·25	1·25	2	1·5
0·00156	0·125	1	1	0·5	0·5	0·5	0·5	0·75	1·25
Nil	Nil (saline control)	0·25		0·25		0·3		0·3	

DISCUSSION

The work described in this paper establishes beyond reasonable doubt that the main component of the third factor involved in the virulence-enhancing action of hog gastric mucin is a heparin: both the chemical and biological properties have been adequately checked.

In addition to its significance in virulence enhancement, the isolation of a crystalline heparin from crude hog gastric mucin has not been reported before. Meyer, Smyth & Palmer (1937) described as the 'acid polysaccharide' of crude hog gastric mucin (Wilson's Laboratories, Chicago) an acetylated mucoitin monosulphuric acid with a negative optical rotation, which was isolated after initial alkaline treatment. Later Meyer & Smyth (1938) and Meyer (1938) described the acid calcium salt of a mucoitin sulphuric acid isolated without alkaline digestion. This contained 9·1 % of sulphur, indicating more sulphate groups than a mucoitin monoassociation with protein to form complexes of varying size was demonstrated also by the virulence-enhancing activities of some samples obtained from an impure batch of third factor in the preparative Spinco ultracentrifuge. Four samples were taken from different depths in the ultracentrifuge tube, and there was no significant difference in their virulence-enhancing activities.

The presence of a highly ionic sulphate group in polysaccharide A and in synthetic detergents, e.g. tergitol (Young & Zelle, 1946; Barnes, 1947), which are also virulence-enhancing, is extremely interesting. It is impossible, however, to attribute the virulence-enhancing activity, or for that matter anticomplementary and anticoagulant activity, solely to the presence of sulphate groups in view of the chemical and biological properties of polysaccharide B (see Smith *et al.* 1952). The more recent chemistry of heparin (Meyer & Schwartz, 1950; Wolfrom *et al.* 1950) has shown the presence of glucosamine-*N*-sulphate groups (—NH—SO_3H),

and the biological activities above may possibly be related to these particular groups.

The fact that a heparin is the main component in the third factor involved in virulence enhancement may give rise to some thought on the wide anatomical occurrence of heparin, and also on its therapeutic use. It must be remembered, however, that heparin is only really effective in enhancing virulence when coupled with the non-specific factors (a particulate residue and a viscous medium), and needs to be combined with polysaccharide C for a maximum effect (see Smith *et al.* 1952). In addition, as has been pointed out before (Smith, 1951*b*), the method of testing virulence-enhancing samples involves a completely artificial infection of mice with *Bact. typhosum* (*Salmonella typhi*) (Smith, 1950*a*) and in no way resembles natural infection. Nevertheless, the presence of heparin in relatively large amounts in such tissues as the lung, liver and spleen (Jorpes, 1946) is worth bearing in mind in any studies on natural infection.

SUMMARY

1. The main component of the third factor involved in the virulence-enhancing action of hog gastric mucin has been obtained essentially homogeneous as judged by electrophoresis, ultracentrifugation and fractional solubility. It has been identified as a heparin by its chemical and biological properties.

2. This is the first isolation of a heparin mucoitin disulphuric acid from crude hog gastric mucin obtained from whole hog mucosae.

3. The significance of these findings is discussed.

Our thanks are due to Dr D. W. Henderson for his interest in this work. We are also indebted to Mr R. Strange for microanalysis and paper chromatography and to Mr S. Peto for statistical analysis of results.

Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper. Photographs are Crown Copyright reserved, reproduced by permission of the Controller, H.M. Stationery Office.

REFERENCES

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1950). *Biochem. J.* **46**, 426.
Barnes, J. M. (1947). *Brit. J. exp. Path.* **28**, 385.
Charles, A. F. & Todd, A. R. (1940). *Biochem. J.* **34**, 112.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Ecker, E. E. & Cross, P. (1929). *J. infect. Dis.* **44**, 250.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Jorpes, E. (1935). *Biochem. J.* **29**, 1817.
Jorpes, E. (1942). *Biochem. J.* **36**, 203.
Jorpes, J. E. (1946). *Heparin in Thrombosis.* Oxford University Press.
Jorpes, J. E., Boström, H. & Mutt, V. (1950). *J. biol. Chem.* **183**, 607.
Jorpes, J. E. & Gardell, S. (1948). *J. biol. Chem.* **176**, 267.
Lefèvre, K. U. & Tollens, B. (1907). *Ber. dtsch. chem. Ges.* **40**, 4513.
Meyer, K. (1938). *Cold Spr. Harb. Sym. quant. Biol.* **6**, 91.
Meyer, K. & Smyth, E. M. (1938). *J. biol. Chem.* **123**, xxxiv.
Meyer, K., Smyth, E. M. & Palmer, J. W. (1937). *J. biol. Chem.* **119**, 73.
Meyer, K. H. & Schwartz, D. E. (1950). *Helv. chim. Acta*, **33**, 1651; *Experientia*, **6**, 332.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Partridge, S. M. (1950). *Biochem. Soc. Symp.* no. 3, 59.
Reinert, M. & Winterstein, A. (1939). *Arch. int. Pharmacodyn.* **62**, 47.
Smith, H. (1950*a*). *Biochem. J.* **46**, 352.
Smith, H. (1950*b*). *Biochem. J.* **46**, 356.
Smith, H. (1951*a*). *Biochem. J.* **48**, 441.
Smith, H. (1951*b*). *J. infect. Dis.* **88**, 207.
Smith, H., Gallop, R. C. & Stanley, J. L. (1952). *Biochem. J.* **52**, 15.
Smith, H., Harris-Smith, P. W. & Stanley, J. L. (1951). *Biochem. J.* **50**, 211.
Wolfrom, M. L., Montgomery, R., Karabinos, J. V. & Rathger, P. (1950). *J. Amer. chem. Soc.* **72**, 5796.
Wolfrom, M. L. & Rice, F. A. H. (1946). *J. Amer. chem. Soc.* **68**, 532.
Wolfrom, M. L. & Rice, F. A. H. (1947). *J. Amer. chem. Soc.* **69**, 2919.
Wolfrom, M. L., Weisblat, D. I., Karabinos, J. V., McNeely, W. H. & McLean, J. (1943). *J. Amer. chem. Soc.* **65**, 2077.
Young, G. A. & Zelle, M. R. (1946). *J. Infect. Dis.* **79**, 266.

ADDENDUM

Physico-chemical Examination of Polysaccharide A

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The polysaccharide, which had been twice purified by precipitation as the neutral barium salt and conversion to the sodium salt, was finally dialysed against water and freeze-dried. It was examined in the electrophoresis apparatus and the ultracentrifuge and diffusion measurements were made at various concentrations by two independent methods. The partial specific volume was also determined. The data obtained lend support to the characterization of the polysaccharide as a heparin. The degree of homogeneity of the material has also been assessed and a value calculated for its molecular weight.

METHODS

Electrophoresis. The experiments were carried out at 0° in the medium size (10 ml.) U-tube of the Tiselius electrophoresis apparatus using the diagonal schlieren optical system. In every case the solutions were dialysed to equilibrium with the buffer used (ionic strength, $I = 0{\cdot}2$). Observations were made at two different pH's, 8·0 and 4·5, and at two concentrations, 2·0 and 0·7 % at each pH. The 2·0 % solutions were examined in order to reveal any traces of contaminating material; those at the lower concentration were used for the calculation of the electrophoretic mobility of the material.

Ultracentrifuge. This part of the investigation was carried out with the 'Spinco' electrically driven ultracentrifuge, fitted with a diagonal schlieren optical system. The experimental technique followed exactly that outlined by Creeth (1952) and Charlwood (1952). The solutions were prepared directly either in phosphate or phosphate-sodium chloride buffer, pH 8·0, total I, 0·2; one run was made at 1·0 % concentration in acetate buffer pH 4·5, $I = 0{\cdot}10$. The rotor speed was 59 780 rev./min. in all experiments. As the sedimentation constant is comparatively low, the distances moved by the boundary are rather small; accordingly between seven and ten photographs of the sedimenting boundary position were obtained at regular intervals in each experiment.

Diffusion. Measurements of the diffusion constant were made by two entirely independent methods: (1) By means of the standard Tiselius electrophoresis apparatus using the diagonal schlieren optical system to determine the concentration distribution. The measurements were carried out at 0°. (2) More accurately, especially in the low concentration range, by the Gouy interferometric diffusion apparatus (Coulson, Cox, Ogston & Philpot, 1948; Creeth, 1952). The measurements in this case were made near 25°.

Partial specific volume. This parameter was determined at 25° in the usual pyknometric manner at two concentrations, 2·0 and 1·0 %, in phosphate buffer, pH 8·0; I, 0·2.

RESULTS

Electrophoresis. Fig. 1 shows the patterns obtained in each of the four experiments carried out. In every case, there appeared to be present, on casual inspection, two components; a major component migrating rapidly to the anode and a minor non-migrating component. By careful removal of this latter 'component' it was possible to show that it was in fact none other than the result of a δ or ϵ boundary, and consisted only of the buffer used. (See Table 1 of the main part of this paper.) The polysaccharide was therefore electrophoretically a single component. It has a high anodic mobility at pH 8·0 and this is not greatly reduced at pH 4·5. These observations fit in well with the chemistry of the material as outlined in the main part of this paper. The highly ionic nature of the material also explains the abnormally large boundary anomalies observed.

Records of similar measurements made on heparin (see Wolfrom & Rice, 1947) point to a behaviour closely resembling that of polysaccharide A, though it would appear probable that the large proportion of 'stationary component' reported by these workers is none other than an enhanced δ and ϵ boundary.

Since the electrophoretic mobility of the polysaccharide may be an important characteristic of the material, mobility values have been calculated in each case from the rate of movement of the cathode descending boundary. From the cross-sectional area of the U-tube and the current passing, the operative potential gradient was computed using the conductivity as measured on the original solution at 0°. For a 0·7 % solution at pH 8·0 the mobility was found to be $1{\cdot}41 \times 10^{-4}$ cm.²/sec./V. A slightly lower value, $1{\cdot}27 \times 10^{-4}$ cm.²/sec./V., was obtained for a 0·75 % solution at pH 4·5 (see Fig. 1). These values fall within the range given for heparin by Wolfrom & Rice (1947).

Sedimentation. At all concentrations and at both pH 4·5 and 8·0, only one, apparently symmetrical, peak was obtained. Fig. 2 shows the experimental record obtained at 1 % concentration.

value $-0{\cdot}778 \pm 0{\cdot}068$ (S.E.). The extrapolated value of the sedimentation constant at zero concentration is then $2{\cdot}25 \pm 0{\cdot}17$ (95 % fiducial limits), the value used in calculating the molecular weight.

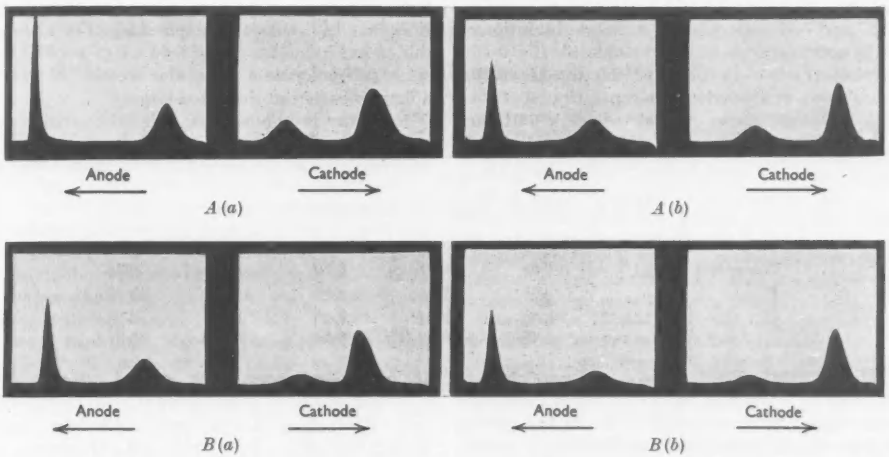

Fig. 1. Electrophoresis patterns of the polysaccharide A. The arrows indicate the direction of migration of the polysaccharide towards the anode. *A*, in phosphate buffer pH 8·0, $I = 0{\cdot}2$; (*a*) 2·0 % solution: exposure after 1 hr. 46 min., current = 9·3 ma., mobility = $1{\cdot}26 \times 10^{-4}$ cm.²/sec./V.; (*b*) 0·7 % solution: exposure after 2 hr. 8 min., current = 6·5 ma., mobility = $1{\cdot}41 \times 10^{-4}$ cm.²/ sec./V. *B*, in acetate buffer pH 4·5, $I = 0{\cdot}2$; (*a*) 2·0 % solution: exposure after 2 hr., current = 5·0 ma., mobility = $1{\cdot}18 \times 10^{-4}$ cm.²/sec./V.; (*b*) 0·7 % solution: exposure after 2 hr., current = 7·4 ma., mobility = $1{\cdot}27 \times 10^{-4}$ cm.²/sec./V.

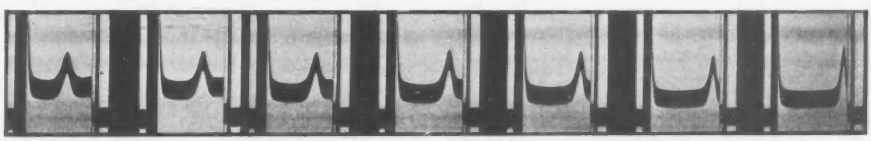

Fig. 2. Sedimentation pattern of polysaccharide A. 1 % concentration in phosphate buffer pH 8·0, $I = 0{\cdot}20$. Speed = 59 780 rev./min. Interval between exposures = 32 min. Migration from right to left.

Table 1. *Sedimentation constants of polysaccharide A*

(Solvent: Phosphate buffer pH 8·0, $I = 0{\cdot}20$, unless otherwise stated.)

Concentration (%)	0·13	0·13	0·28	0·30	0·40	0·47	0·60	1·0	1·0*
S_{20}	2·10	2·18	2·08	1·96	1·98	1·94	1·75	1·47	1·58

* In this experiment, the solvent was sodium acetate, pH 4·5, $I = 0{\cdot}10$.

The sedimentation constant of the substance is markedly dependent on concentration, as is shown by the results summarized in Table 1.

All S_{20} values are given in Svedberg units and have been reduced to sedimentation in water.

The S_{20} values in phosphate buffer are approximately linearly related to concentration, the slope of the line expressing the regression of the sedimentation constant on concentration having the value $-0{\cdot}778 \pm 0{\cdot}068$ (S.E.). The extrapolated value of the sedimentation constant at zero concentration is then $2{\cdot}25 \pm 0{\cdot}17$ (95 % fiducial limits), the value used in calculating the molecular weight. The limits of error of this value, 4·9 %, are somewhat higher than the corresponding limits observed with albumins (Creeth, 1952; Charlwood, 1952); this reflects the greater difficulty in dealing with substances of lower sedimentation constant, especially when this shows an appreciable variation with concentration.

Diffusion. All diffusion constants were corrected for viscosity and temperature to diffusion in water

at 20°, and the coefficients are quoted in units of 10^{-7} cm.^{2} sec.$^{-1}$.

The diagonal schlieren patterns obtained with 1·5 and 0·70 % concentrations of the polysaccharide (undialysed) in pH 8·0 phosphate buffer, $I = 0{\cdot}20$, were somewhat asymmetric, and yielded D_A values of 7·87 and 7·47 respectively. A dialysed solution of 0·70 % concentration in sodium chloride ($I = 0{\cdot}10$), phosphate ($I = 0{\cdot}10$), buffer at pH 8·0 (total $I = 0{\cdot}20$), gave almost symmetrical concentration distributions, yielding mean values of $D_A = 6{\cdot}91$ and $D_M = 6{\cdot}63$. These values are consistent with those obtained with the Gouy diffusiometer (Table 2).

The molecular weight of 16 500 may be compared with the value of about 20 000 for the sodium salt of heparin, quoted by Wolfrom, Montgomery, Karabinos & Rathger (1950), though this figure is not of much significance if it has been calculated from diffusion measurements alone on the assumption that the molecule is spherical. For example, taking our value for D_{20} of $6{\cdot}66 \times 10^{-7}$ we find for a spherical molecule a molecular weight of 163 000, a figure nearly ten times too large.

It has not been possible in this brief investigation to give any estimate of the degree of polydispersity of the polysaccharide. In common with its class (see, for example, Record, 1948; Record & Stacey, 1948) a certain degree of polydispersity is to be expected, but a study of the sedimentation diagrams suggests that in this case it is probably not extensive.

Table 2. *Diffusion coefficients for polysaccharide A*

(V = refraction increment of polysaccharide in wavelengths of retardation.)

Concentration (%)	0·28	0·31	0·40	0·51	0·56
V	6·96	7·78	9·88	12·73	13·85
D_{20}	6·63	6·64	6·39	6·63	6·77
	6·68	6·53	6·54	6·75	6·81

There is thus little apparent dependence of the diffusion coefficient on concentration over the lower concentration range. In view of the greater accuracy and consistency of the Gouy method with dilute solutions (Creeth, 1952) the mean value of the results in Table 2, $D_{20} = 6{\cdot}66 \pm 0{\cdot}09$ (S.D.) has been used for the subsequent calculation of the molecular weight.

DISCUSSION

The fact that only one component is apparent in both electrophoresis and sedimentation, at two widely separated pH's, adds considerable weight to the evidence that the polysaccharide A consists of a single well defined molecular species.

Combining the values of the sedimentation and diffusion constants with the value of 0·50 obtained for the partial specific volume, the figure of 16 500 is obtained for the molecular weight of the substance.

The frictional ratio, f/f_0, has a value of 2·15; this corresponds to an axial ratio of 25 if the molecule is assumed to be an unhydrated rod.

SUMMARY

1. The polysaccharide A is homogeneous both in electrophoresis and in sedimentation.

2. The molecular weight calculated from the diffusion coefficient ($6{\cdot}66 \times 10^{-7}$), the sedimentation constant, $C \to 0$ ($2{\cdot}25 \times 10^{-13}$) and the partial specific volume (0·50) is 16 500, the frictional ratio 2·15 pointing to marked molecular asymmetry.

3. The electrophoretic mobility of the material is $1{\cdot}41 \times 10^{-4}$ cm.^{2}/sec./V. in pH 8·0 phosphate and $1{\cdot}27 \times 10^{-4}$ cm.^{2}/sec./V. in pH 4·5 acetate buffer, being anodic in each case. The electrophoretic behaviour is that of a highly ionized molecule.

Acknowledgement is due to Mr K. H. Grinstead for technical assistance.

REFERENCES

Charlwood, P. A. (1952). *Biochem. J.* **51**, 113.
Coulson, C. A., Cox, J. T., Ogston, A. C. & Philpot, J. St L. (1948). *Proc. roy. Soc.* A, **192**, 382.
Creeth, J. M. (1952). *Biochem. J.* **51**, 10.
Record, B. R. (1948). *J. chem. Soc.* p. 1567.
Record, B. R. & Stacey, M. (1948). *J. chem. Soc.* p. 1561.
Wolfrom, M. L., Montgomery, R., Karabinos, J. V. & Rathger, V. (1950). *J. Amer. chem. Soc.* **72**, 5796.
Wolfrom, M. L. & Rice, F. A. H. (1947). *J. Amer. chem. Soc.* **69**, 2918.

The Oxidation of Nicotinic Acid by *Pseudomonas fluorescens*

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The ability of certain bacterial species to oxidize nicotinic acid was first reported by Allinson (1943) who used suspensions of a *Pseudomonas* culture originally isolated by Dubos & Miller (1937). Nicotinic acid was degraded by suspensions prepared from cultures grown only in the presence of this compound. These suspensions failed to attack nicotinamide, coramine or nicotinuric acid. Allinson concluded that this oxidation was an adaptive feature of the organism.

A short time later, Koser & Baird (1944) isolated strains of *Pseudomonas fluorescens* and *Serratia marcescens* which were able to grow in a nicotinic acid medium. These strains did not attack *iso*-nicotinic acid, picolinic acid or pyridine. There was no growth or poor growth on nicotinamide, quinolinic acid or ethyl nicotinate. Nicotinethylamide and nicotindiethylamide supported growth to a very slight extent.

Nichol & Michaelis (1947) suggested that an oxidase was responsible for the destruction of the nicotinic acid. Certain inhibitors were able to interfere drastically with the ability of bacterial suspensions to utilize nicotinic acid. These included sodium azide, hydroxylamine hydrochloride, octan-2-ol, laurylpyridinium chloride and toluene.

The experiments reported below were designed to elucidate further, by means of Warburg respiration technique, the utilization of nicotinic acid as a source of carbon and nitrogen by *Ps. fluorescens*. A number of nicotinic acid analogues were tested with a view to finding intermediates and thereby outlining the possible metabolic route. In addition, inhibitors were used to characterize the enzyme system.

EXPERIMENTAL

Organism. The organism used was *Ps. fluorescens* 30 of Koser & Baird (1944), which was found by Nichol & Michaelis (1947) to be the most active of the strains tested.

Media and suspensions. The broth medium, the agar slopes and suspensions of resting bacterial cells were prepared as outlined by Nichol & Michaelis (1947). In the media, apart from the agar, the only source of C and N was nicotinic acid.

Measurement of oxidation. Conventional Warburg respiration technique was used for the measurement of O_2 uptake at 27°. The total volume of reacting liquid in each flask was 2·3 ml., consisting of 0·3 ml. of cell suspension in distilled water, 0·1–1·0 ml. of the substrate under test, 0·5 ml. of 0·2 M-phosphate buffer, pH 7·0, and distilled water to make up to volume. The centre well contained 0·2 ml. of 20% (w/v) KOH. The rate of shaking was 120–150 complete excursions/min. The gas space was filled with air and readings were taken at 15 min. intervals.

Determination of nicotinic acid. Nicotinic acid was determined by the method described by Nichol (1946). This is essentially a modification of Allinson's (1943) method. The difference in chromogen content (compounds reacting with CNBr) of an enzyme-treated sample as compared to one untreated, gives an accurate measure of the nicotinic acid content. The following procedure was used. Two liquids were treated in the same manner, except that one sample had 0·1 ml. distilled water replacing 0·1 ml. of cell suspension used in the other sample. Cell suspension (0·1 ml.), prepared as described, or distilled water and 0·5 ml. of 0·2 M-phosphate buffer, pH 6·0, were added to 5 ml. of test solution. After standing at room temperature for 30 min., the mixtures were centrifuged at high speed. Samples (1 ml.) of CNBr (freshly prepared by shaking successive portions of powdered KCN with saturated bromine water until the colour disappeared) were added to 5 ml. of the above supernatant and held at 60° for 7–10 min. The test tubes were cooled to 20° and held at this temperature for 5 min. after which 1 ml. of 5% (w/v) aqueous *p*-phenylenediamine was added. The solutions were mixed by inversion and 0·5 ml. of 20% (w/v) HCl was immediately added. Readings were made in a Coleman Universal spectrophotometer at 420 mμ. The difference in optical densities of the enzyme-treated and the enzyme-untreated samples formed the basis for the calculation of the nicotinic acid content.

RESULTS

The stoicheiometric relationship between oxygen consumption and nicotinic acid breakdown. By the use of Warburg technique, a series of experiments was carried out with varying amounts of nicotinic acid as substrate. It was assumed that respiration on nicotinic acid came to an end at that point where the curve of oxygen consumption against time flattened out. The total oxygen consumptions at these points were plotted against the μmoles of nicotinic acid originally in the substrates. The results are shown in Fig. 1. The points obtained lie close to a straight line and show that the degradation of 1 mole of nicotinic acid was accompanied by the

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uptake of 3·9 moles of oxygen. This value is distinctly higher than that of 2·7 reported by Nichol & Michaelis (1947).

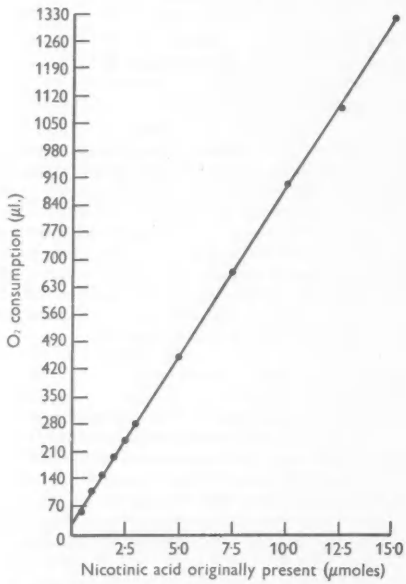

Fig. 1. O_2 consumption by *Pseudomonas fluorescens* with varying quantities of nicotinic acid as substrate. The side arms of each manometer vessel contained the amounts of nicotinic acid listed. The main chamber contained 0·5 ml. 0·2 M-phosphate buffer, pH 7·0, 0·3 ml. cell suspension, and water to make up 2·3 ml. total volume excluding KOH. The centre well contained 0·2 ml. 20% (w/v) KOH. Temp., 27°; gas, air. Values shown were obtained after the substrate was exhausted.

As shown subsequently (Table 1), the ratio of oxygen consumed to nicotinic acid degraded gradually rose and did not reach the maximal figure until all of the nicotinic acid had disappeared. Nichol & Michaelis (1947) determined the stoicheiometric ratio before the autorespiration stage had been reached and when nicotinic acid was still present in the substrate. Therefore they could not have obtained the final stoicheiometric relationship reported here. It appears likely that they would have obtained the higher ratio had they allowed the oxidation to go to completion.

The relationship between oxygen consumption and degradation of nicotinic acid. In the following Warburg experiment, the nicotinic acid in the substrate was $2{\cdot}17 \times 10^{-3}$ M. At intervals, a pair of flasks was removed and one drop of a 1% (w/v) aqueous solution of anhydrous mercuric chloride was added to each flask in order to stop any further reaction. The suspension in each flask was centrifuged at 10 000 rev./min. and 1 ml. of the supernatant was diluted to 100 ml. with distilled water. The nicotinic acid content of the resultant solution was determined as previously described. Table 1 gives the results obtained.

The results show that the molar ratio between oxygen consumed and nicotinic acid oxidized gradually increased during the time the cell suspension was respiring, and the maximum was obtained only when the substrate was completely oxidized. Apparently the experiment was stopped before all the intermediates were oxidized so that even though no nicotinic acid was detectable, the molar ratio had not reached 3·9 as obtained in other experiments.

We may also conclude from these results that the first stage in the degradation of nicotinic acid requires less than 1 mole of oxygen per mole of nicotinic acid. The first determinations were made after 5 min. but it seems likely that had a determination been made earlier, a ratio of one atom of oxygen per mole of nicotinic acid would have been obtained.

Hale, Davis & Baldwin (1942) have shown that the cyanogen bromide reaction is given by all α-unsubstituted pyridine compounds. The failure of the nicotinic acid residue to react with cyanogen

Table 1. *Ratio of oxygen consumed to nicotinic acid destroyed by* Pseudomonas fluorescens *as respiration proceeds*

(The main chamber of the manometer vessels contained 0·5 ml. 0·2 M-phosphate buffer, pH 7·0, 0·3 ml. cell suspension and 1·3 ml. water. The centre well contained 0·2 ml. 20% (w/v) KOH; 0·2 ml. 0·025 M-nicotinic acid was in the side arm. Temp., 27°; gas, air.)

Nicotinic acid destroyed (μmoles)	Percentage of total nicotinic acid	Corresponding O_2 consumption (μmoles)	Percentage of ultimate O_2 consumption	Molar ratio O_2 consumed to nicotinic acid oxidized
2·46	49	1·92	11	0·78
2·70	54	2·22	12	0·82
3·94	79	4·64	25	1·18
4·61	92	5·99	33	1·30
4·67	93	10·42	57	2·23
4·67	93	14·42	79	3·09
5·00	100	18·25	100	3·65

bromide is therefore due to α-substitution or the opening of the pyridine ring. Experiments described subsequently indicate that carbon dioxide is produced early in the oxidation. In addition, several analogues of nicotinic acid where the pyridine ring was intact were relatively inert. The evidence, therefore, strongly suggests that the initial oxidation involves opening of the pyridine ring. The major part of the oxygen consumption apparently involved the oxidation of products of degradation.

Table 2. *Respiratory quotients of* Pseudomonas fluorescens *on a nicotinic acid substrate*

(Manometric technique involving the 'direct method' for CO_2 was employed. The side arm of each vessel contained 0·2 ml. 0·025 M-nicotinic acid. The main chamber contained 0·5 ml. 0·2 M-phosphate buffer, pH 7·0, 0·3 ml. cell suspension and 1·3 or 1·5 ml. water. The centre well contained 0·2 ml. 20% (w/v) KOH or was empty. Temp., 27°; gas, air.)

Time (min.)	O_2 consumed (μl.)	CO_2 produced (μl.)	R.Q.
15	66·9	38·2	0·57
30	151·9	89·2	0·59
45	251·9	181·8	0·72
60	366·6	294·5	0·80
75	489·9	419·9	0·86
100	534·1	477·6	0·89
115	543·3	490·6	0·90

Respiratory quotient. The respiratory quotient of a cell suspension on nicotinic acid substrate was determined by manometric technique using the 'direct method' for carbon dioxide. The results obtained are listed in Table 2. The calculations were made on the basis of the substrate remaining at pH 7·0. Since the bicarbonate errors in the calculations of carbon dioxide output are large and increase as the pH rises, the values obtained are most probably too low, especially for the later parts of the experiment. It has been found that under the experimental conditions, respiring suspensions of *Ps. fluorescens* on nicotinic acid become alkaline, the pH rising beyond 7·8. The final respiratory quotient value of 0·90 most probably approaches unity.

From the above data, the relationship at maximum oxidation, of 3·5 moles of carbon dioxide produced per mole of nicotinic acid destroyed, is obtained. It must again be emphasized that this value is minimal and most likely approaches 3·9. The results therefore agree approximately with those expected if four of the six carbon atoms of the nicotinic acid molecule form carbon dioxide.

The evolution of carbon dioxide at an early stage in the oxidation cannot be attributed to decarboxylation because pyridine will react with cyanogen bromide whereas the product obtained failed to react.

Anaerobic activity. Measurements of anaerobic activity were made by the method of Quastel & Wheatley (1938). There appeared to be no anaerobic degradation of nicotinic acid, thus corroborating the work of Nichol (1946) who used Thunberg technique. It is clear, therefore, that the initial attack on the nicotinic acid molecule does not take place in the absence of oxygen, and this phase of the degradation of nicotinic acid must be a specific oxidative action.

The effects of cell suspensions on other compounds in the presence and absence of nicotinic acid. The compounds used included those with the pyridine ring, indole derivatives and aromatic diketones. Table 3 gives the results obtained with the pyridine analogues used.

The effect of the organism on compounds containing the indole nucleus, especially tryptophan, was determined because of the numerous references in the literature that tryptophan was a precursor of nicotinic acid in the metabolism of a number of genera (Haskins & Mitchell, 1949; Rosen, Huff & Perlzweig, 1946). Table 4 gives the results obtained with indolylpropionic acid and with L-tryptophan.

Table 3. *Effects of suspensions of* Pseudomonas fluorescens *on various pyridine derivatives*

(The side arms of each manometer vessel contained either one of the compounds listed below or 0·2 ml. 0·025 M-nicotinic acid or both. The main chamber contained 0·5 ml. 0·2 M-phosphate buffer, pH 7·0, 0·3 ml. cell suspension and water to make up 2·3 ml. excluding KOH. The centre well contained 0·2 ml. 20% (w/v) KOH. Temp., 27°; gas, air. All values corrected for autorespiration.)

Compound	Final concentration (mM)	Duration of exp. (min.)	O_2 uptake (μl.) With compound	With nicotinic acid	With both	Change (%)
2-Aminopyridine	2·17	100	3·7	401·7	467·9	16·5
3-Aminopyridine	2·17	115	3·7	523·6	483·4	−7·7
Coramine	2·17	75	8·3	389·1	453·7	16·6
Nicotinamide	8·54	140	13·8	388·6	391·6	0·8
Nicotine	0·677	135	0·9	366·4	414·6	13·2
Picoline	5·04	170	7·6	380·3	422·4	11·1
Pyridine	2·16	150	10·3	378·7	432·4	14·2
Pyridoxin	1·28	90	1·0	352·3	387·9	10·1
Sodium pyridine-3-sulphonate	4·35	90	7·2	430·3	430·3	0
Trigonelline	2·17	100	12·6	401·7	491·7	22·4
Sodium 3-sulphanilamidopyridine	3·43	90	2·1	430·3	388·9	−9·6

It may be seen that the oxygen consumption of the suspension in the presence of any of the above compounds (pyridine and indole analogues) when used as the sole substrate, was negligible. In all cases where the oxygen consumption in the presence of any of the above compounds and nicotinic acid was greater than the oxygen consumption on the latter substrate alone, the increase was only evident after the nicotinic acid had been completely exhausted.

Table 4. *Effects of suspensions of* Pseudomonas fluorescens *on* L-*tryptophan and indolylpropionic acid*

(The side arms of each manometer vessel contained either one of the compounds listed below or 0·2 ml. 0·025 M-nicotinic acid or both. The main chamber contained 0·5 ml. 0·2 M-phosphate buffer, pH 7·0, 0·3 ml. cell suspension and water to make up 2·3 ml. excluding KOH. The centre well contained 0·2 ml. 20% (w/v) KOH. Temp., 27°; gas, air. All values corrected for autorespiration.)

Compound	Final concentration (mM)	Duration of exp. (min.)	O_2 uptake (μl.) With compound	With nicotinic acid	With both	Change (%)
L-Tryptophan	2·17	115	8·9	523·6	519·1	0·9
Indolylpropionic acid	2·17	90	13·2	356·1	424·8	19·3

Table 5. *Influence of cyclic diketones and quinol on the oxygen consumption of* Pseudomonas fluorescens *on a nicotinic acid substrate*

(The side arms of each manometer vessel contained either one of the compounds listed below or 0·2 ml. 0·025 M-nicotinic acid or both. The main chamber contained 0·5 ml. 0·2 M-phosphate buffer, pH 7·0, 0·3 ml. cell suspension and water to make up 2·3 ml. total volume excluding KOH. The centre well contained 0·2 ml. 20% (w/v) KOH. Temp., 27°; gas, air.)

Compound	Final concentration (M)	Duration of exp. (min.)	O_2 consumption (μl.) Auto-respiration	With compound	With nicotinic acid	With both	Inhibition (%)
Benzoquinone	$4{\cdot}10 \times 10^{-3}$	105	20·3	19·3	473·5	20·6	99·9
Benzoquinone	$8{\cdot}60 \times 10^{-4}$	120	5·1	—	427·5	307·9	28·3
Quinol	$3{\cdot}06 \times 10^{-3}$	105	20·3	15·4	473·5	490·1	0
Quinhydrone	$8{\cdot}93 \times 10^{-4}$	105	20·3	9·8	473·5	27·6	98·4
1:2-Naphthoquinone	$1{\cdot}30 \times 10^{-4}$	120	5·1	—	427·5	67·7	85·2
1:4-Naphthoquinone	$3{\cdot}30 \times 10^{-4}$	120	5·1	—	427·5	17·0	97·2

Quinones. The first diketone tested was isatin which was selected because it had the indole nucleus in its structure. This compound inhibited the respiration of a cell suspension on a nicotinic acid substrate when added in a concentration of $1{\cdot}75 \times 10^{-3}$ M. When the isatin was added before the nicotinic acid, inhibition was more marked. This inhibition seemed to be of a competitive nature since an increased concentration of nicotinic acid raised the oxygen consumption when the isatin concentration remained constant.

Since indole was inactive, it appeared that the inhibitory effect of the isatin might be attributed to the ketone groups in the molecule. In order to verify this assumption, experiments were conducted using a number of aromatic diketones as well as quinol as listed in Table 5.

The oxygen consumption, when any one of these compounds was the sole organic substrate, was less than the autorespiration. Whereas the diphenol was inert, the corresponding diketone was a very effective inhibitor.

The above effect was eliminated by the addition of D-cysteine hydrochloride. With $1{\cdot}85 \times 10^{-3}$ M-benzoquinone and $8{\cdot}35 \times 10^{-4}$ M-cysteine the oxygen consumption was 83% of normal. When the benzoquinone was added 15 min. before the cysteine, the inhibition was not neutralized. In the concentrations used, the cysteine appeared to neutralize the inhibiting effect of the benzoquinone when the molar ratio of the former to the latter was 1:2. Displacement of this equilibrium either way caused pronounced inhibition. When cysteine was $4{\cdot}5 \times 10^{-3}$ M and benzoquinone was $3{\cdot}28 \times 10^{-3}$ M, the resulting inhibition was 68·7%.

The effects of other inhibitors. A number of other inhibitors were tested for their effects on the nicotinic acid-oxidizing ability of the cell suspension and gave the results listed in Table 6.

The 2:2′-dipyridyl was dissolved in 10% (v/v) ethanol because it was insoluble in water. Other experiments have shown that the ethanol itself has no inhibitory action but supports cell respiration to a considerable extent. The inhibition caused by the 2:2′-dipyridyl is therefore solely due to this compound.

From the results it is evident that the enzyme studied involves the participation of a metal.

DISCUSSION

The stoicheiometric relationship between nicotinic acid oxidized and oxygen consumed indicates that the oxidation of the nicotinic acid molecule proceeds to over 70% of total theoretical oxidation. The relationship found was 3·9 moles of oxygen consumed per mole of nicotinic acid degraded. By calculation, if the nitrogen of the nicotinic acid molecule forms ammonia; the hydrogen, water; and the carbon, carbon dioxide; 11 atoms of oxygen would be required for complete oxidation. The value of 7·8 atoms indicates over 70% total oxidation. Experiments in this laboratory have shown that ammonia is produced early in the reaction so that the nitrogen of the nicotinic acid molecule undoubtedly forms this product.

The respiratory quotient was 0·90 in the later stages of the respiration. As previously indicated, this value is minimal and most probably approached unity. Therefore only two carbon atoms of the nicotinic acid molecule form unknown products; the other four form carbon dioxide.

The fact that there is apparently no reaction under anaerobic conditions supports the work of Nichol & Michaelis (1947). We may conclude that the initial attack on the nicotinic acid molecule is essentially an oxidative action and that the enzyme responsible is a true oxidase. The relationship between nicotinic acid oxidation and oxygen consumption—as the oxidation proceeds—supports the above assumption. A ratio of 0·78 mole of oxygen per mole of nicotinic acid was obtained in the first 5 min. Most likely 1 atom of oxygen was used to degrade the nicotinic acid molecule to the point where it no longer gave the cyanogen bromide reaction. The evidence strongly supports the assumption that this initial attack involved the opening of the pyridine ring.

The work with the nicotinic acid analogues has clearly emphasized the specific nature of the oxidase. If the carboxyl group of the nicotinic acid molecule is treated to form the amide, or replaced by a sulphonic acid group, an amino group or a sulphanilamide group or removed entirely, the enzyme is inactive on the resulting compound. The work of Koser & Baird (1944) indicates that the carboxyl group must be in the 3 position.

The interpretation of the results according to Stanier's (1947) theory of simultaneous adaptation showed that none of the pyridine or indole compounds tested was involved in any of the intermediate steps in the degradation of nicotinic acid. Here we have additional evidence for the conclusion that the first step in the oxidation of the nicotinic acid molecule is the opening of the pyridine ring.

Any increased oxygen consumption when the substrate was nicotinic acid and any one of the above compounds, apparently was due to interaction between these compounds and degradation products of the nicotinic acid molecule. There could have been no adaptation to any of these substrates because the compound alone supported virtually no cell respiration and active cell suspensions were used in all cases.

The enzyme system studied here appears to have no connexion at all to the system in some species which converts tryptophan to nicotinic acid.

The experiments where known inhibitors were used indicate that a metal took part in the enzyme action.

Attempts to isolate active cell-free preparations were unsuccessful. No components could be

Table 6. *Effects of certain inhibitors on the oxygen consumption of* Pseudomonas fluorescens *on a nicotinic acid substrate*

(The side arms of each manometer vessel contained either one of the inhibitors listed below or 0·2 ml. 0·025 M-nicotinic acid or both. The main chamber contained 0·5 ml. 0·2 M-phosphate buffer, pH 7·0, 0·3 ml. cell suspension and water to make up 2·3 ml. total volume excluding KOH. The centre well contained 0·2 ml. 20% (w/v) KOH. Temp., 27°; gas, air.)

Compound	Final concentration (M)	Duration of exp. (min.)	O_2 consumption (μl.) Auto-respiration	With compound	With nicotinic acid	With both	Inhibition (%)
Dichloroacetic acid	$2{\cdot}60 \times 10^{-2}$	90	18·6	—	406·2	17·4	100
2:2′-Dipyridyl	$2{\cdot}44 \times 10^{-3}$ in 10% (v/v) ethanol	90	7·2	107·0	364·5	187·3	68·8
Iodoacetic acid	$2{\cdot}17 \times 10^{-4}$	90	7·0	—	227·4	63·2	74·5
8-Hydroxyquinoline	$1{\cdot}10 \times 10^{-3}$	135	11·6	15·7	378·0	66·5	85·0
Sodium cyanide	$4{\cdot}80 \times 10^{-3}$	90	18·6	—	406·2	53·4	91·0
Disodium hydrogen arsenite	$1{\cdot}87 \times 10^{-4}$	120	29·3	—	541·8	331·7	41·0
Sodium fluoride	$2{\cdot}17 \times 10^{-3}$	75	18·3	—	329·7	325·7	1·3
Sodium diethyl-dithiocarbamate	$2{\cdot}50 \times 10^{-3}$	75	48·7	29·8	292·5	99·1	83·4
Sodium azide	$6{\cdot}69 \times 10^{-2}$	90	13·3	—	286·5	47·8	87·4

dialysed away when cell suspensions were dialysed in a cellophan sausage skin in running tap water and in distilled water. Ultrasonic irradiation at 350 kcyc./sec. under an atmosphere of air, carbon dioxide or nitrogen resulted in inactive suspensions. The decrease in activity apparently paralleled the rupturing of the cells. Allinson (1943) and Nichol & Michaelis (1947) were also unsuccessful in obtaining active cell-free enzyme preparations.

SUMMARY

1. Cell suspensions of *Pseudomonas fluorescens* oxidized nicotinic acid, consuming 3·9 molecules of oxygen per molecule of nicotinic acid attacked.

2. The respiratory quotient was low at the start of the oxidation but rose to 0·90 towards the end of the respiration.

3. The degradation of the nicotinic acid did not take place under anaerobic conditions. The enzyme involved in the initial attack on the molecule is therefore a true oxidase.

4. The evidence obtained very strongly supports the conclusion that the first step in the degradation of the nicotinic acid molecule is the opening of the pyridine ring.

5. The specificity of the enzyme has been studied. If the carboxyl group of the nicotinic acid molecule is treated to form the amide, or replaced by a sulphonic acid group, an amino group, or a sulphanilamide group, or removed entirely, the enzyme is inactive on the resulting compound.

6. Indolylpropionic acid and L-tryptophan were not attacked.

7. Aromatic diketones and cysteine inhibited nicotinic acid oxidation. The effect of one could be neutralized by the other at specific concentration ratios.

8. The enzyme studied contains a non-dialysable metal.

9. Attempts to obtain active cell-free enzyme preparations were unsuccessful.

The above work is based on the thesis submitted by A. Pinsky in partial fulfilment of the requirements for the Ph.D. degree, May 1949. During part of the work he was holder of a studentship from the National Research Council (Canada) to whom thanks are expressed.

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REFERENCES

Allinson, M. J. C. (1943). *J. biol. Chem.* **147**, 785.
Dubos, R. & Miller, B. F. (1937). *J. biol. Chem.* **121**, 429.
Hale, E. B., Davis, G. K. & Baldwin, H. R. (1942). *J. biol. Chem.* **146**, 553.
Haskins, F. A. & Mitchell, H. K. (1949). *Proc. nat. Acad. Sci., Wash.*, **35**, 500.
Koser, S. A. & Baird, G. R. (1944). *J. infect. Dis.* **75**, 250.
Nichol, C. A. (1946). *A study of an enzymatic method for the determination of nicotinic acid in foods.* M.Sc. Thesis, McGill University.
Nichol, C. A. & Michaelis, M. (1947). *Proc. Soc. exp. Biol., N.Y.*, **66**, 70.
Quastel, J. H. & Wheatley, A. H. M. (1938). *Biochem. J.* **32**, 936.
Rosen, F., Huff, J. W. & Perlzweig, W. A. (1946). *J. biol. Chem.* **163**, 343.
Stanier, R. Y. (1947). *J. Bact.* **54**, 339.

o-Phenylenediamine and 1:2-Diamino-4-nitrobenzene as Reagents for α-Keto Acids

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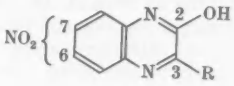
It is possible to isolate and characterize as 2:4-dinitrophenylhydrazones many of the keto acids formed by *Penicillium chrysogenum* and thus to study the mould's intermediary metabolism. These hydrazones are, however, sometimes neither adequately stable nor chromatographically separable.

The condensation of *o*-phenylenediamine (OPD) with α-ketonic acids has been shown to yield stable quinoxalinols (cf. Hinsberg, 1887), which were found to be separable by paper chromatography. These derivatives were most useful, but it was thought that the introduction of a chromogenic grouping might make chromatography easier. We chose 1:2-diamino-4-nitrobenzene (DANB) in preference to a diazo derivative, as this might involve an increase in molecular size such as to swamp the effect of differences in the original

keto acids. The products are compounds of the general formula I. The position of the nitro group (x, 6 or 7) has not yet been determined.



I

EXPERIMENTAL

Synthesis of nitroquinoxalinol derivatives of α-ketoacids

2-Hydroxy-3-methyl-x-nitroquinoxaline. To 110 mg. pyruvic acid (1·25 m-mole) in 10 ml. water was added a solution of 190 mg. (1·25 m-mole) DANB in 10 ml. 2N-HCl. After 5 min. a precipitate appeared, and after 2 hr. the resulting quinoxalinol was filtered off and dried (yield 160 mg.). The product was twice recrystallized from hot ethanol-water; m.p. 245°. (Found: C, 52·5; H, 3·5; N, 20·3. $C_9H_7N_3O_3$ requires C, 52·7; H, 3·4; N, 20·5%.)

2-Hydroxy-3-isopropyl-x-nitroquinoxaline. To 145 mg. dimethylpyruvic acid (1·25 m-mole) in 10 ml. water were added 10 ml. of a solution of 190 mg. DANB in 10 ml. 2N-HCl. After 5 min. the product began to precipitate. After 2 hr. it was filtered off and dried (yield 190 mg.). The product was twice recrystallized from hot ethanol; m.p. 257°. (Found: C, 57·1; H, 4·9; N, 17·8. $C_{11}H_{11}N_3O_3$ requires C, 56·7; H, 4·7; N, 18·0%.)

2-Hydroxy-3-phenyl-x-nitroquinoxaline. To 188 mg. benzoylformic acid (1·25 m-mole) in 10 ml. water were added 10 ml. of a solution of 190 mg. DANB in 10 ml. 2N-HCl. There was an immediate precipitate. After 2 hr., it was filtered off and dried (yield 231 mg.). The product was twice recrystallized from hot ethanol; m.p. 264°. (Found: C, 63·1; H, 3·6; N, 15·3. $C_{14}H_9N_3O_3$ requires: C, 62·9; H, 3·4; N, 15·7%.)

2-Hydroxy-3-(2-carboxyethyl)-x-nitroquinoxaline. To 182 mg. α-ketoglutaric acid (1·25 m-mole) in 10 ml. water were added 10 ml. of a solution of 190 mg. DANB in 10 ml. 2N-HCl. After 10 min. a precipitate began to form slowly. After 2 hr. it was filtered off and dried (yield 280 mg.). The product was twice recrystallized from a 1:4 mixture of ethyl acetate and light petroleum (b.p. 60–80°); m.p. 204°. (Found: C, 50·2; H, 3·8; N, 15·9. $C_{11}H_9N_3O_3$ requires C, 50·2; H, 3·4; N, 16·0%.)

Separation of quinoxalinols by paper chromatography

Large sheets of Whatman no. 4 paper were cut into 30 mm. strips perpendicular to the 'grain'. About 50 μg. of derivative (from OPD or DANB) were applied in about 20 μl. of ethyl acetate or $CHCl_3$, about 60 mm. from the end of the paper. The papers were hung, by means of small (6 mm.) 'bull-dog' clips, from the rubber stoppers of 500 ml. graduated cylinders, so as to dip into 25 ml. of solvent therein. They were developed at room temperature until the solvent front had risen into the top tenth of the strip. The strips were removed and air-dried at room temperature.

Location of spots. After drying, the NDB derivatives were visible as pale-yellow spots in daylight or, more clearly, as dull-brown spots against a purplish background under ultraviolet light. The yellow colour could also be accentuated by spraying with a 2% ethanolic solution of KOH.

The OPD derivatives were colourless in daylight, but showed clearly as yellowish fluorescent patches under ultraviolet light. In addition, spraying with a 0·1% aqueous solution of 2-chloro-4-nitrophenyldiazonium naphthyl-2-sulphonate (Heinrich & Schuler, 1947, 1948) gave a pink spot with the derivative from pyruvic acid, although no coloured compound seemed to be formed by that from dimethylpyruvic, benzoylformic or α-ketoglutaric acids. This reaction was presumably due to coupling on the methyl group.

Chromatography of nitroquinoxalinols on alumina

The columns were made up by transferring a slurry of 30 g. of alumina (Peter Spence and Co., Grade H) in $CHCl_3$ into 16 mm. (internal diameter) tubes and allowing them to drain slowly, with occasional tapping to give a depth of about 130 mm.

About 60 mg. of the mixture of nitroquinoxalinols were applied in 2–3 ml. ethyl acetate. Development was carried out with the following succession of solvent mixtures: $CHCl_3$, $CHCl_3$-ethanol mixtures (9:1, 4:1, 1:1, 1:4), ethanol, ethanol-water mixtures (9:1, 3:1, 1:1, 1:3) and ethanol-aqueous N-NaOH (1:3). If reasonably fast movement of the front band was obtained, the same solvent was continued until complete elution ('break-through') was obtained. The loading of these columns may be increased to about 50 mg. of each component, with some deterioration in sharpness of separation.

RESULTS

The nitroquinoxalinols from pyruvic acid, dimethylpyruvic acid, ketoglutaric acid and benzoylformic acid were synthesized as described in the experimental section.

Paper chromatographic separation of OPD derivatives of α-ketoacids

The quinoxalinols derived from pyruvic, dimethylpyruvic and α-ketoglutaric acids were found to be separable by filter paper chromatography (Table 1).

With the ammoniacal solvents the α-ketoglutarate spot was remarkably clear and compact.

Paper chromatographic separation of DANB derivatives of α-keto acids

The nitroquinoxalinols derived from pyruvic, dimethylpyruvic and α-ketoglutaric acids could be readily separated by paper-strip chromatography. The derivative from benzoylformic acid was found to streak badly with most solvents.

The best separations were achieved with dry or aqueous ethanol or methanol (Table 2). The following solvents were found to be poor or useless: *n*-butanol, *sec.*-butanol and their water mixtures, chloroform, benzene, ethyl acetate and their mixtures with various light petroleums, ether and wet ether. Benzene and toluene were ineffective when run on papers saturated with formamide, propylene glycol or 60% aqueous ethanol as the stationary phase.

The more water in the solvent, the faster was the travel of the materials but the greater the streaking.

Chromatography on alumina of DANB derivatives of keto acids

A mixture of about 20 mg. each of methyl-, *iso*-propyl- and carboxyethyl-nitroquinoxalinols in about 2 ml. ethyl acetate were applied to an alumina column as described earlier. *iso*Propyl-nitroquinoxalinol moved slowly with chloroform alone, but 100 ml. of chloroform-ethanol (4:1) mixture washed the band right off the column. Methylnitroquinoxalinol only began to move down slightly with ethanol and was completely washed off by 110 ml. of ethanol-water (9:1). Only the alkaline solution moved the carboxyethyl derivative which came right through with 40 ml. The spread of the bands before leaving the column was about 20 mm.

Table 1. *Paper chromatography (ascending) of OPD derivatives of ketoacids*

Solvent	Methanol	Ethanol	*n*-Butanol saturated with 1·5N-NH_4OH	*sec.*-Butanol-1·5N-NaOH (4:1)	Amyl alcohol saturated with 1·5N-NH_4OH
Duration of run (hr.)	2·5	4	20	20	20
Distance traversed by solvent (cm.)	21·2	21·6	27·4	24·5	25·2
R_f values of quinoxalinols from:					
α-Ketoglutaric acid	0·39	0·10	0·22	0·33	0·02
Pyruvic acid	0·62	0·72	>0·90	>0·90	>0·90
Dimethylpyruvic acid	0·78	0·87	>0·90	>0·90	>0·90

Table 2. *Paper chromatography of NDB derivatives of keto acids (ascending)*

Solvent	Methanol	Methanol-water (9:1 v/v)	Methanol-water (7:3 v/v)	Ethanol	Ethanol-water (9:1 v/v)	Ethanol-water (7:3 v/v)
Duration of run (hr.)	4	4	4	4	4	4
Distance traversed by solvent (cm.)	27	26·5	26·0	27·2	27·0	26·3
R_f values of nitroquinoxalinols from:						
α-Ketoglutaric acid	0·43	0·55	0·75	0·32	0·55	0·72
Pyruvic acid	0·59	0·60	0·75	0·63	0·77	0·80
Dimethylpyruvic acid	0·73	—	—	0·80	—	—

DISCUSSION

The advantages of 1:2-diamino-4-nitrobenzene over 2:4-dinitrophenylhydrazine as a reagent for α-keto-acids are the following: (1) the reaction is more specific for α-keto groups; (2) theoretically, the quinoxalinols, since they contain conjugated heterocyclic ring systems, should be much more stable than the hydrazones; (3) the chromatographic separation of characterizable quantities is easier than for the hydrazones, which still possess carboxylic acid groups, rendering alumina separations difficult; (4) the melting points of the compounds are reasonably sharp and have a range adequate for differentiation by this means.

Only in one category are the hydrazones more useful, namely in qualitative examination, where paper chromatography on aqueous ammoniacal alcoholic mixtures (*n*-butanol, *sec.*-butanol, amyl alcohol, *n*-hexanol, *sec.*-octanol (after Cavallini, Frontali & Toschi, 1949)) gives a very clear distinction between derivatives. Even here, the DANB derivatives are useful in corroboration, or where speed is an urgent consideration.

The OPD derivatives are reasonably useful, but suffer from their lack of colour and failure generally to couple with diazo reagent. Their chromatographic separation is not so clear as that of DANB.

SUMMARY

1. A series of stable nitroquinoxalinols, new coloured derivatives of α-keto acids, has been prepared.

2. The quinoxalinols obtained by condensation of either *o*-phenylenediamine or 1:2-diamino-4-nitrobenzene with pyruvic, dimethylpyruvic and α-ketoglutaric acids, may be separated by paper chromatography.

3. The nitroquinoxalinols may also be separated on alumina.

4. 1:2-Diamino-4-nitrobenzene is superior to 2:4-dinitrophenylhydrazine as a reagent for α-keto acids, in specificity, stability, separability and characterizability of the products.

The authors wish to thank Mrs F. M. Goodinson for technical assistance.

REFERENCES

Cavallini, D., Frontali, N. & Toschi, G. (1949). *Nature, Lond.*, **163**, 568.

Heinrich, P. & Schuler, W. (1947). *Helv. chim. Acta*, **30**, 886.

Heinrich, P. & Schuler, W. (1948). *Helv. chim. Acta*, **31**, 320.

Hinsberg, O. (1887). *Liebigs Ann.* **237**, 340.

The Metabolism of Succinic Acid in the Rumen of the Sheep

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Succinic acid is a major end product of two cellulose-fermenting bacteria isolated in pure culture from the rumen of cattle and sheep. *Ruminococcus flavefaciens*, isolated by Sijpesteijn (1948, 1949, 1951), produced some 0·5 m-mole succinic acid per m-mole cellulose (calculated as glucose) fermented. *Bacteroides succinogenes*, isolated from the bovine rumen by Hungate (1950), was found by this author to produce 51·3 mg. succinic acid from 82 mg. cellulose. Since large amounts of cellulose are fermented each day in the rumen and in view of the importance of the two organisms mentioned above it is reasonable to expect to find a high concentration of succinate in the rumen; however, Marston (1948) found that only very small amounts of succinate were produced during the fermentation of cellulose in an artificial rumen.

On the other hand, propionic acid is known to be formed in large quantities when cellulose is fermented *in vitro* (Elsden, 1945; Marston, 1948), and there is now evidence that propionic acid is formed by the decarboxylation of succinic acid (Johns, 1951*a*, *b*, *c*; Delwiche, 1948). A possible solution to this paradox is that succinic acid is formed in the rumen from cellulose and is rapidly decarboxylated to propionic acid.

The present investigations deal with the production of succinic acid in the rumen during the digestion of a normal diet and with the fate of succinic acid added directly to the rumen. In addition, the metabolism of succinic acid by washed suspensions of rumen bacteria has been investigated. This use of washed suspensions of rumen bacteria represents a new experimental approach to the study of the rumen, and some indications are given of the possible applications of this technique.

METHODS

Design of animal experiments. Scottish black-faced wethers fitted with permanent rumen fistulae were used as experimental animals. All animal experiments were commenced some 20 hr. after the last meal and, unless otherwise stated, only water was provided during the period of the experiment, the cage having been cleared of surplus food the previous evening. An initial sample of rumen contents was withdrawn via the fistula and prepared for analysis; 1 hr. later a second sample was withdrawn and the test substance then given. Thereafter samples of rumen contents were withdrawn at appropriate time intervals.

Preparation of rumen contents for analysis. The rumen contents were filtered through muslin and 25 ml. of the liquor pipetted into a 50 ml. centrifuge tube; 3 ml. of 4 N-H_2SO_4 were added to stop further metabolic changes and also to facilitate the subsequent analyses; the pH was below 1·2. The mixture was then centrifuged and the clear supernatant used for the analyses.

Estimation of volatile fatty acids. Total volatile acids were separated by steam distillation in the apparatus described by Markham (1942). An appropriate sample of the acidified liquor was placed in the apparatus, the steam turned on and the acid content of the distillate determined by titration with 0·02 N-NaOH in a stream of CO_2-free air. For the estimation of the individual acids, the distillate obtained from the Markham apparatus was re-distilled according to the procedure of Friedemann (1938). The distillate so obtained was made alkaline to phenol red, evaporated to dryness and analysed by the silica gel partition chromatogram (Elsden, 1946). As in a previous publication (Elsden, 1945) the acids moving faster than butyric acid were collected together with the butyric acid, and the whole fraction for convenience referred to as butyric acid.

Succinic acid estimation. The manometric method described by Krebs (1937) was used throughout. We found, however, that under our conditions the oxidation of succinate was very slow and at least 2 hr. were required for complete oxidation. This was in marked contrast to the experience of Krebs (1937) who found the reaction to be complete in 40 min. This difference in behaviour has been traced to the K_2HPO_4 component of our stock buffer mixture, and all samples of K_2HPO_4 that we have been able to test have shown the same inhibitory action. On the other hand, the following buffer mixtures had no effect on the oxidation of succinate by the minced-muscle preparation:

$KH_2PO_4 + KOH$; $KH_2PO_4 + Na_2HPO_4$; $HCl + Na_2HPO_4$ and $Na_2HPO_4 + HCl + 0{\cdot}1$ N-KCl,

and we are forced to conclude that the samples of K_2HPO_4 available commercially contain a substance or substances which are inhibitory to the succinic dehydrogenase system. In our experience to date this is the only enzyme system inhibited by the K_2HPO_4 preparations.

Preparation of washed suspensions of rumen bacteria. Rumen contents obtained fresh from the animal via the fistula were filtered through muslin and the liquor centrifuged for 1 min. at 1000 rev./min. in the M.S.E. 'Major' (Measuring and Scientific Equipment Ltd., London, S.W. 1) centrifuge using the angle head. This serves to remove the

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smaller food particles, the Protozoa and some of the larger bacteria. The supernatant liquid was carefully decanted and centrifuged at 3500 rev./min. for 30 min. The deposit so obtained was then suspended in freshly boiled out 0·05 M-phosphate buffer, pH 6·5, containing in addition 0·02% (w/v) $Na_2S.9H_2O$ to maintain a low rH, and then recentrifuged. The deposit was then suspended in a suitable buffer containing 0·02% (w/v) $Na_2S.9H_2O$, the volume of which equalled that of the original filtrate. It is essential to minimize the exposure of the organisms to the air; failure to do this results in a marked reduction in the activity of the final suspension. This is borne out not only by our own experience but also that of Mr K. Shazly (private communication) who has found that active deamination of amino-acids by washed suspensions of rumen bacteria only occurs if these precautions are adhered to.

Warburg manometers were used for small-scale experiments. Large-scale experiments were carried out in 100 ml. Erlenmeyer flasks fitted with a centre well and a side bulb of 4 ml. capacity. The flask and the side bulb were closed with rubber stoppers each carrying a stopcock. When N_2 was used as the gas phase it was freed from O_2 by passage over copper turnings, coated with palladium and heated to about 400° in an electric furnace. Activities are expressed as $Q_{CO_2} = \mu$l. CO_2/mg. dry wt. of suspension/hr.

RESULTS

Formation of succinate in the rumen. To test whether succinate is produced in the rumen during digestion of our normal stock diet an animal was given 2 lb. of chopped oat straw and meadow hay plus 0·5 lb. of flaked maize; it was allowed to feed for a period of 90 min. after which the uneaten residue was removed. The samples of rumen contents taken during the course of the experiment were analysed for total volatile acids and for succinate. The results are given in Table 1. The initial concentration of succinate was low and over the experimental period there was a slight rise followed by a fall. The increase in succinate concentration was insignificant when compared with the increase in total volatile acids.

Table 1. *Production of succinic acid and volatile fatty acids in the rumen during digestion*

(At 61 min. 2 lb. of chaffed oat straw and meadow hay plus 0·5 lb. flaked maize given and the animal allowed to feed for 90 min.)

Time of sampling (min.)	Succinic acid (m-mole/100 ml.)	Volatile acids (m-equiv./100 ml.)
0	0·022	6·67
60	0·054	6·30
150	0·363	10·45
250	0·066	12·6
330	0·046	11·9

The fate of succinate added directly to the rumen. Succinic acid (25 g.) was added to about 300 ml. distilled water and the pH adjusted to 6·5 with 40% (w/v) NaOH. The volume was made up to 400 ml. and after the initial samples had been taken the solution was added directly to the rumen via the fistula. Further samples were taken over a period of 5 hr. and analysed for acetate, propionate, butyrate and succinate. The results are given in Fig. 1. It will be seen that the succinate disappeared within 5 hr. and at the same time the amount of propionate increased. On the other hand, the amounts of acetate and butyrate present showed little change. The suggestion is, therefore, that succinate is rapidly converted to propionate in the rumen.

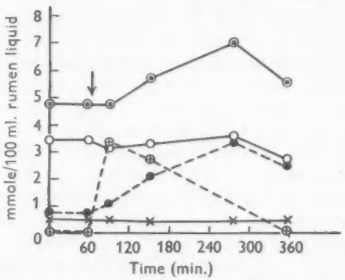

Fig. 1. Effect of addition of 25 g. succinic acid as sodium salt, to the rumen. ⊕, succinic acid; ⊙, total volatile acids; ○, acetic acid; ●, propionic acid; ×, butyric acid.

Metabolism of succinate by washed suspensions of rumen bacteria. A detailed study of the decomposition of succinate could not be carried out under *in vivo* conditions and we sought for a procedure which would allow us to work *in vitro*. Our results suggested that succinic acid is converted to propionic acid by a decarboxylation, and hence it seemed desirable that the *in vitro* technique should be such as to enable us to follow the reaction manometrically. We therefore examined the possibility of using washed suspensions of the rumen bacteria, and it was soon apparent that such preparations were most suitable for our purpose. When succinate was incubated with a washed suspension of rumen bacteria under an atmosphere of O_2-free N_2 there was a steady output of CO_2 and the production of a steam-volatile acid. Table 2 gives the results of a quantitative experiment. If the control values are deducted it will be seen that 1 mole of succinate gives rise to 1 mole of CO_2 and 1 equivalent of volatile acid, which would be the case if succinate were quantitatively decarboxylated to propionic acid and CO_2. The optimum pH of the reaction was found to be about pH 6·0 (Table 3), in general agreement with Johns (1951*a*).

The amount of volatile acid produced in the manometric experiments was insufficient for a positive identification of the propionic acid so the experiment was repeated on ten times the scale of

the previous experiment, using the large vessels. Two such vessels were used, each containing 20 ml. of washed suspension; to the first was added 0·204 m-mole succinic acid as the sodium salt in 2 ml. distilled water and to the second, which served as a control, 2 ml. distilled water. At the end of the experiment 3 ml. of 3N-H_2SO_4 were added to each flask and the contents steam-distilled and the total volatile acids determined. 0·229 m-equiv. volatile acid was formed in the presence of succinate and 0·028 m-equiv. in the control. Thus the net formation of volatile acid was 0·201 m-equiv. from 0·204 m-mole of succinate. The neutralized distillate from the succinate flask was redistilled according to Friedemann (1938) and the distillate prepared for analysis on the silica gel chromatogram. Two bands were observed: one, very faint, had the same R_F as acetic acid and the other, which was the major component, had the same R_F as propionic acid. The acetic acid was presumably the endogenous volatile acid and the propionic acid was produced from the succinate, a fact in keeping with our earlier observations.

Table 2. *Decarboxylation of succinate by a washed suspension of rumen bacteria*

(Experiment carried out in Warburg manometer with double side-bulb cups. 0·3 ml. substrate or 0·3 ml. distilled water in one side bulb, and 0·3 ml. 3N-H_2SO_4 in the other. Suspension made up in 0·05M-phosphate buffer, pH 6·5 containing 0·01% (w/v) $Na_2S.9H_2O$; 2 ml. suspension per cup. Total CO_2 determined by addition of acid at the end of the experiment. Gas phase N_2; temp. = 37°; time = 160 min.)

Exp.	Succinate added (μmole)	CO_2 produced (μmole)	Volatile acid produced (μequiv.)
1	20·4	22·7	22·5
2	0	2·6	4·0

Table 3. *Effect of* pH *on decarboxylation of succinate by a washed suspension of rumen bacteria*

(Manometer cups made up as follows: suspension (in 0·9% (w/v) NaCl containing 0·01% (w/v) $Na_2S.9H_2O$), 2 ml.; 0·2M-phosphate buffer, 2·0 ml.; sodium succinate, 0·4 ml. (0·04 m-mole) in side bulb 1; 4N-H_2SO_4 (0·3 ml.) in side bulb 2; gas phase N_2; temp. = 37°. At zero time succinate added and after 17 min. acid added to stop the reaction and to liberate bound CO_2. All figures corrected for CO_2 produced in control experiments without substrate.)

pH	5·7	5·9	6·05	6·25	6·55	7·4
Q_{CO_2}	55·6	58·6	60·5	55·3	52·6	41·3

In addition to the decarboxylation of succinic acid we have made some preliminary observations on the metabolism of other substances by these preparations. Glucose and cellobiose are readily fermented with the production of acid and some CO_2. The time course of a typical experiment is given in Fig. 2; Table 4 gives the quantitative data of the same experiment. It will be noted that the amount of acid is small, approximately 1 equiv. is produced per hexose unit. Our colleague, Dr Dyfed Lewis (Lewis, 1951*a*), has examined the fermentation of glucose in more detail and has found that, in addition to lactic acid, acetic and propionic acids are produced in quantity along with smaller amounts of *n*-butyric acid and traces of *n*-valeric and *n*-caproic (hexanoic) acids. When the fermentations were carried out in phosphate buffer with a CO_2-absorber in the centre well, the pressure change was no greater than in the control, indicating that little or no gas other than CO_2 was produced.

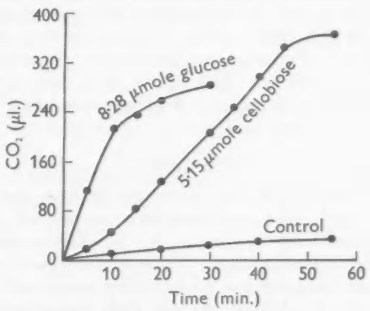

Fig. 2. Fermentation of glucose and cellobiose by a washed suspension of rumen bacteria. Suspending medium: 0·01M-$NaHCO_3$ containing 0·002M-KH_2PO_4 and 0·01% (w/v) $Na_2S.9H_2O$.

Table 4. *Fermentation of glucose and cellobiose by a washed suspension of rumen bacteria*

(Experiment carried out in Warburg manometer with double side-bulb cups. Suspension made up in 0·01M-$NaHCO_3$ solution containing 0·002M-KH_2PO_4 and 0·01% (w/v) $Na_2S.9H_2O$. 2 ml. suspension (1·1 mg. total N) used per cup, with 0·3 ml. substrate in side bulb 1 and 0·3 ml. 2N-H_2SO_4 in side bulb 2. Gas phase = N_2 + 5% CO_2 with sticks of yellow phosphorus in the centre wells; temp. = 38°. Duplicate set of manometers used for the estimation of initial HCO_3^-. All quantities in μmole.)

	Glucose	Cellobiose
Substrate	8·28	5·15
Initial HCO_3^-	20·0	20·0
Final HCO_3^-	10·3	8·8
Acid produced	9·7	11·2
Total CO_2	12·8	16·2
Free CO_2	3·1	5·0
μmole acid/μmole substrate	1·17	2·15

During the course of our studies of the decarboxylation of succinate we had occasion to use H_2 instead of N_2 as the gas phase. In the presence of H_2 it was found that the usual evolution of CO_2 soon stopped and thereafter a gas uptake occurred. This gas uptake did not occur if the centre well contained

a CO_2-absorber, indicating that both H_2 and CO_2 were involved in the reaction. When washed suspensions of rumen bacteria were suspended in 0·01 M-sodium bicarbonate and incubated under $H_2 + CO_2$ (95:5 by vol.), a vigorous consumption of gas occurred which persisted for a number of hours. Whilst it has been impossible to exclude completely the formic hydrogenlyase system (Woods, 1936), determination of the amount of sodium bicarbonate present at the beginning and the end of the experiment showed that the amount of acid formed was far less than the amount of gas consumed; whereas, had the formic hydrogenlyase system been responsible 1 equiv. of acid would have been produced for every molecule of H_2 consumed. It was not possible with the apparatus available to determine both the H_2 and CO_2 utilized, but recently Lewis (1951*a*), using the Summerson manometer (Summerson, 1939), has shown that the H_2:CO_2 ratio greatly exceeds the 1:1 ratio required by the formic hydrogenlyase system.

DISCUSSION

Our experiments show conclusively that under *in vitro* and *in vivo* conditions succinic acid is rapidly converted to propionic acid by the micro-organisms of the sheep's rumen. There is consequently no conflict between the metabolic studies with pure cultures of cellulose-fermenting bacteria carried out by Sijpesteijn (1948, 1949, 1951) and by Hungate (1950) and the *in vitro* investigations of Elsden (1945) and Marston (1948); and the conversion of cellulose to propionic acid in the rumen proceeds in part, at least, via succinic acid. The fact that during the digestion of a normal meal, and in the presence of an active succinic decarboxylase, small amounts of succinic acid accumulate, suggests that large quantities of this compound are produced during the digestive process. From the rate of succinate disappearance from the rumen, and assuming that the rumen contains 6 l. of liquid and that the liquid contains 1% (w/v) dry weight of micro-organisms, it may be calculated that the *in vivo* process has a Q_{CO_2} of about 15 as compared with the values of 20–60 obtained in our *in vitro* experiments.

The question is now raised, which of the types of organism in the rumen are responsible for the decarboxylation reaction? Two groups of bacteria have been reported to decarboxylate succinic acid: *Veillonella gazogenes* (Johns, 1951*a*, *b*) and species of the genus *Propionibacterium* (Delwiche, 1948; Johns, 1951*c*). Of these *V. gazogenes* is the more active ($Q_{CO_2} = 300$ as opposed to $Q_{CO_2} = 4$ for the propionibacteria). Johns (1951*a*) reports that the rumen contains at least 6×10^6 *V. gazogenes*/ml. of rumen liquor. This is not a particularly large number, but clearly part at least of the decarboxylation reaction must be due to this organism. Elsden (1945) reported the isolation of species of propionibacteria from the rumen of the sheep and the ox. There is now reason to believe that these organisms are not present in the rumen in large numbers, and even if they were, the slowness with which they decarboxylate succinic acid would suggest that they play no significant part in this reaction in the rumen.

The pH optimum of the decarboxylation was found both by Johns (1951*a*) and by ourselves to be about pH 6·0, and since the pH of the rumen at the height of digestion is also in this range (Phillipson, 1942) the conditions in the rumen are optimal for the conversion of succinic acid to propionic acid.

Johns has also, quite independently, introduced the use of washed suspensions of rumen bacteria for the study of the decarboxylation reaction (Johns, 1951*a*). His method of preparation of the washed suspensions differed significantly from our own, and it is not surprising therefore to find differences in behaviour. Thus, whereas Johns (1951*a*) found the pH curve for the decarboxylation to have a sharp optimum about pH 6·0, we obtained a much broader pH curve. Further, Johns (1951*a*) records Q_{CO_2} values of 130, whereas our values for the same reaction—the decarboxylation of succinic acid—were in the range 20–60. The main difference between the two methods of preparing the washed suspensions is to be found in the preliminary centrifugal treatment of the crude rumen liquor. Thus Johns (1951*a*) centrifuged rumen liquor for 5 min. and used the supernatant for the preparation of the suspension, whereas we centrifuged for only 1 min. and at a lower speed. It is probable therefore that our suspensions contained a much greater weight of bacteria, not all of which would be active, than did those of Johns, a factor which would tend to reduce the Q_{CO_2}. In addition, Johns's animals had been fed on pasture, whereas our animals were fed a diet of hay and flaked maize and it is not unlikely that the composition of the population differed considerably.

The study of the function of the rumen population under *in vitro* conditions has many advantages over the *in vivo* method; but, at the same time, the *in vitro* methods which have been used to date are open to criticism. Thus the incubation period was usually such that extensive growth occurred and, in the presence of a simple substrate, a highly selected population could and probably would develop bearing little quantitative relation to that found in the rumen. Further, even if the experiments are of short duration the blank activity of crude rumen liquor is high unless the animal has been fasted for a considerable time, and under these conditions the population changes. Nor is it possible to study the metabolism of a single substrate under controlled conditions owing to the presence of food particles and volatile fatty acids.

The application of the washed-suspension technique to the study of the rumen population was foreshadowed by the work of Marston (1948) though he incubated his suspensions for 24 hr.—sufficient time for extensive growth to occur. Under our conditions the time of an experiment is kept to a minimum, and growth sufficient to produce a significant alteration in the population is unlikely. Our preparations thus appear to offer many advantages over the previous methods. In particular, the blank metabolism is so low that it is possible to utilize manometric methods and associated analytical techniques, which permit a more detailed analysis of single reactions than would otherwise be possible. In support of this claim we may cite not only our own work but also that of Lewis (1951*a*, *b*) on the reduction of nitrate and sulphate by rumen bacteria. Clearly, however, the washed suspension method must not be used to the exclusion of all others. It is, indeed, most important that wherever possible, *in vitro* findings should be confirmed by *in vivo* experiments.

Despite its advantages the use of washed suspensions is not free from objections. First, it is questionable whether the composition of the washed suspension is quantitatively the same as the population of the rumen, even neglecting the Protozoa which are removed by the preliminary centrifugation. In addition, the fractionation is arbitrary and seldom sharp and we have frequently observed that a considerable amount of the bacterial fraction other than Protozoa is removed during the first brief centrifugation. Further, a significant proportion of the population is concerned with the fermentation of such insoluble materials as cellulose, hemicellulose, polyuronides and starch, and it is probable that many of these organisms will be attached to the particulate matter removed during the preparation of the suspension. Finally, it is always possible that the more delicate organisms are inactivated during the preparation of the suspension. Thus it is probable that our final suspension is deficient in the larger bacteria, in organisms attacking insoluble materials and in the more delicate bacteria.

It appears to us that the washed-suspension technique could be readily adapted to the study of other bacterial populations, and in particular to the study of the organisms of the alimentary tract of animals other than herbivores. Such an approach to the study of bacteria of the human colon and large intestine might well provide useful information on the role of micro-organisms in both normal and pathological conditions.

SUMMARY

1. The concentration of succinate in the rumen of sheep is low and there is only a slight increase after feeding.

2. Added succinate is rapidly removed from the rumen and at the same time there is an increase in the concentration of propionate but not of any other volatile fatty acid.

3. Washed suspensions of rumen bacteria convert succinate quantitatively to propionate and carbon dioxide.

4. Washed suspensions of rumen bacteria ferment glucose and cellobiose rapidly with the production of about 1 equivalent of acid per hexose unit. In addition some free carbon dioxide is produced.

The cost of this work was borne by the Agricultural Research Council. One of us, A.K.S., wishes to express her indebtedness to the British Council for a scholarship.

REFERENCES

Delwiche, E. A. (1948). *J. Bact.* **56**, 811.
Elsden, S. R. (1945). *J. exp. Biol.* **22**, 51.
Elsden, S. R. (1946). *Biochem. J.* **40**, 252.
Friedemann, T. E. (1938). *J. biol. Chem.* **123**, 161.
Hungate, R. E. (1950). *Bact. Rev.* **14**, 1.
Johns, A. T. (1951*a*). *J. gen. Microbiol.* **5**, 317.
Johns, A. T. (1951*b*). *J. gen. Microbiol.* **5**, 326.
Johns, A. T. (1951*c*). *J. gen. Microbiol.* **5**, 337.
Krebs, H. A. (1937). *Biochem. J.* **31**, 2095.
Lewis, D. (1951*a*). Thesis. Sheffield University.
Lewis, D. (1951*b*). *Biochem. J.* **49**, 149.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Marston, H. R. (1948). *Biochem. J.* **42**, 564.
Phillipson, A. T. (1942). *J. exp. Biol.* **19**, 186.
Sijpesteijn, A. K. (1948). Thesis. Leiden.
Sijpesteijn, A. K. (1949). *Antonie v. Leeuwenhoek*, **15**, 49.
Sijpesteijn, A. K. (1951). *J. gen. Microbiol.* **5**, 869.
Summerson, W. H. (1939). *J. biol. Chem.* **131**, 579.
Woods, D. D. (1936). *Biochem. J.* **30**, 515.

Studies on Cholinesterase

8. DETERMINATION OF REACTION VELOCITY CONSTANTS WITH A REVERSIBLE INHIBITOR OF PSEUDO-CHOLINESTERASE

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According to the classical theory of enzyme-substrate kinetics as proposed by Michaelis & Menten (1913), the activity of an enzyme is dependent on, and directly proportional to, the concentration of an intermediary enzyme-substrate complex. The concentration of this complex (ES) is determined by the mass law principles governing a reversible reaction between free enzyme (E_f) and free substrate (S_f), this reaction being of the form

$$E_f + S_f \rightleftharpoons ES.$$

Similarly, it is postulated that the degree of inhibition of enzyme activity by a reversible competitive inhibitor (I_f) is proportional to the concentration of an intermediary enzyme-inhibitor complex which is formed by the reaction

$$E_f + I_f \rightleftharpoons EI.$$

Straus & Goldstein (1943) and Goldstein (1944) have analysed the kinetics of enzyme-substrate-inhibitor reactions from this theoretical viewpoint, and predicted that the relative enzyme concentration could play an important role in determining the mechanism of these reactions. These theoretical predictions have been confirmed experimentally (Myers, 1952), and to this extent the theory not only describes the known facts but can also be used to make a successful prediction.

However, it has been pointed out by Briggs & Haldane (1925) that the overall reaction is actually of the form

$$E_f + S_f \rightleftharpoons ES \rightarrow E_f + \text{reaction products},$$

and that this complete equation must be considered in theoretical treatments of enzyme kinetics. This formulation has been applied to the kinetics of peroxidase activity by Chance (1943), and in this case it was shown that the velocity constant for breakdown of the substrate in the enzyme-substrate complex is much larger than the velocity constant for dissociation of this complex. However, the results with this enzyme system were of a somewhat different nature than is usual with esterases in that the rate of enzymic breakdown of substrate was proportional to the concentration of a third reactant in the system, the oxygen acceptor. It was of interest, therefore, to determine the individual velocity constants involved in a reaction with the cholinesterase system.

Direct measurements of these velocity constants is not possible with an enzyme such as cholinesterase, but in the treatment of enzyme kinetics given by Goldstein (1944), methods were introduced for the indirect determinations of the various velocity constants of each of the individual reactions involved in the above equations. However, it was impossible to arrive at any definite value for enzyme concentration in the cholinesterase system with which the theoretical data were illustrated. Consequently the reaction constants themselves could not be accurately evaluated at that time, and, moreover, the equations given by Goldstein (1944) require some modifications in view of the theoretical considerations of Briggs & Haldane (1925). It has recently been possible to obtain a reliable and relatively accurate estimation of pseudo-cholinesterase concentration by the use of potent reversible competitive inhibitors (Myers, 1952). Knowing the enzyme concentration, definite values can be assigned to all of the determinable reactions constants.

THEORETICAL

The theoretical formulations given below represent a modification and extension of Goldstein's treatment and therefore the mathematical symbols used by Goldstein have been retained throughout. One exception is made to distinguish the velocity constants for the destruction of inhibitor and of substrate, these being designated as k_5 and k_6 respectively. For a comprehensive background to this work, see Goldstein (1944).

In the treatment presented by Goldstein, it has been assumed that the enzyme reactions can be adequately represented by the equations

$$E_f + I_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \quad \text{and} \quad E_f + S_f \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES,$$

where enzyme activity is dependent upon the concentration of ES and inhibition of enzyme activity is dependent upon the concentration of EI. The symbols E, I and S are used to designate the molar concentrations of enzyme, inhibitor, and substrate, respectively. Thus the theoretical equation for the progress of the reaction between enzyme and substrate can be written as

$$\frac{d(ES)}{dt} = k_3 E_f S_f - k_4 ES. \qquad (1)$$

At equilibrium, when the concentration of the enzyme-substrate complex is constant,

$$\frac{d(ES)}{dt}=0, \quad \text{and} \quad \frac{E_t S_t}{ES}=\frac{k_4}{k_3}=K_s.$$

In this case, K_s could be termed a 'dissociation constant'.

The overall reaction between enzyme and substrate is actually of the form

$$E_t+S_t \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES \xrightarrow{k_6} E_t+\text{reaction products},$$

when the initial constant velocity of enzyme activity is being measured (Briggs & Haldane, 1925). Thus the correct equation for the progress of the reaction between enzyme and substrate will be

$$\begin{aligned}\frac{d(ES)}{dt}&=k_3 E_t S_t-k_4 ES-k_6 ES\\&=k_3 E_t S_t-(k_4+k_6)\,ES. \qquad (2)\end{aligned}$$

In this case $K_s=\dfrac{(k_4+k_6)}{k_3}$, where K_s is now the experimental 'Michaelis constant'. Only if k_6 were very small in comparison to k_4 could these two values of K_s be taken as identical. However, the work of Chance (1943) on peroxidase has shown that, in this case at least, the Michaelis constant $(k_4+k_6)/k_3$ is quite different from the dissociation constant k_4/k_3.

The values $k_4=0{\cdot}32$ min.$^{-1}$ and $k_6>3500$ min.$^{-1}$ given by Goldstein (1944) indicate very definitely that k_6, the destruction constant, must also be taken into consideration when working with pseudo-cholinesterase. But the interpretation of these results is further complicated by the fact that one of these values must be erroneous. A consideration of the Eqns. (1) and (2) above will show that the mathematical treatments proposed by Goldstein are still valid if the correct constant (k_4+k_6) is substituted for k_4 throughout these equations and that the experimental value given for 'k_4' must, therefore, be the value of (k_4+k_6). This leaves us with the problem of reconciling the value $(k_4+k_6)=0{\cdot}32$ min.$^{-1}$ with the value $k_6>3500$ min.$^{-1}$.

The value of (k_4+k_6) was determined simultaneously with the value of 'k_2' (or more correctly k_2+k_5) from experimental data for the displacement of acetylcholine from psuedo-cholinesterase by added eserine. The equation representing the change in fractional activity a with time t will be

$$\frac{da}{dt}=\frac{S'(1-a)-I'a}{\dfrac{I'}{(k_4+k_6)}+\dfrac{S'}{(k_2+k_5)}}. \qquad (3)$$

(See Goldstein's equation 15A.) From the data given by Goldstein, $I'=I/K_I$ had the values 35·5 and 355, while $S'=S/K_s$ was constant at 64·4. Thus, using the value $(k_2+k_5)=0{\cdot}026$, $S'/(k_2+k_5)=2500$. For $(k_4+k_6)=0{\cdot}32$, $I'/(k_4+k_6)=111$ and 1110 which, in comparison to the value of $S'/(k_2+k_5)$, would be large enough to affect the value of da/dt appreciably, especially for the higher concentration of inhibitor. But if (k_4+k_6) had a very high value, let us say >3500, then the value of $I'/(k_4+k_6)$ would be negligible in comparison to $S'/(k_2+k_5)$, and thus (k_4+k_6) could not be determined from this type of experiment.

Integration of Eqn. (3), setting $a=1$ when $t=0$, gives

$$a=\frac{1}{S'+I'}\left\{S'+I'\exp\left[-\frac{(S'+I')\,t}{I'/(k_4+k_6)+S'/(k_2+k_5)}\right]\right\}. \qquad (4)$$

(See Goldstein's equation 16A.) On plotting the theoretical curves calculated from this equation, it is found, in fact, that the scattered experimental points can be fitted just as well by an equation where the term $I'/(k_4+k_6)$ is neglected as by an equation where this term has the finite values given by Goldstein. This is illustrated in Fig. 1.

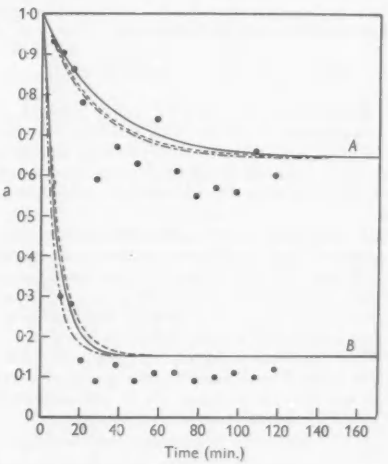

Fig. 1. Displacement of substrate by inhibitor. Eserine added at zero time to an equilibrated mixture of dog serum and acetylcholine. Data of fractional activity of enzyme (a) taken from table IV of Goldstein (1944). Points near A, with $1{\cdot}1\times10^{-6}$M- and points near B, with $1{\cdot}1\times10^{-5}$M-eserine. Theoretical curves calculated from Eqn. (4), see text. – – –, $(k_2+k_5)=0{\cdot}026$, $(k_4+k_6)=0{\cdot}32$ min.$^{-1}$ (identical with curves of fig. 8 of Goldstein, 1944); –·–·–, $(k_2+k_5)=0{\cdot}026$, $(k_4+k_6)>3500$; ——, $(k_2+k_5)=0{\cdot}020$, $(k_4+k_6)>3500$.

A similar criticism also applies to the simultaneous determination of (k_4+k_6) and (k_2+k_5) from the experimental data for displacement of eserine by acetylcholine (Goldstein, 1944). Actually, Goldstein has noted in this case that the 'theoretical treatment does not correctly describe the reaction at low inhibitor concentrations'; but then the assumption that 'free enzyme is negligible' does not apply to the theoretical curves of Fig. 9 in Goldstein's paper. A more correct treatment of the kinetics of displacement of inhibitor by substrate is evident from consideration of the experimental fact that cholinesterase activity reaches a maximum within a very short time after addition of acetylcholine.

When the measurement of the normal activity of a cholinesterase is begun 1–3 min. after the addition of substrate to the enzyme, it is commonly found that the activity is linear in relation to time from the beginning of the determination (reference to this linearity will be found in, for example, Easson & Stedman, 1936; Goldstein, 1944;

Myers, 1951, 1952; Berry, 1951). In other words, the cholinesterase activity has increased from zero and has become constant and maximal within 1–3 min. after the enzyme and substrate were mixed. It is not feasible to measure the rate of increase of activity during the first minute by the techniques commonly used, but we can calculate on a theoretical basis how quickly this must occur. The theoretical equation for the rate of combination of enzyme with substrate, as derived from Eqn. (2), will be

$$\frac{da}{dt} = k_3[S - a\,(S + K_s)].$$

Integrating and setting $a = 0$ when $t = 0$,

$$a = \frac{S'}{S'+1}\{1 - \exp[-(k_4+k_6)\,(S'+1)\,t]\}. \qquad (5)$$

Even using the value $(k_4+k_6) = 0{\cdot}32$ min.$^{-1}$ it is evident that the combination of enzyme with substrate will proceed very rapidly; if $S' = 64$, the enzyme activity is 98% of the maximum it will reach at this specific concentration of substrate within 0·2 min. after the enzyme and substrate are mixed.

Thus we can consider the combination of substrate with free enzyme to be instantaneous under these conditions, especially in view of the fact that the correct value of (k_4+k_6) is probably greater than 3500 min.$^{-1}$. The relative concentration, a, of free enzyme in an equilibrated enzyme-inhibitor system will be given by the equation $I' = (1-a)/a$, or $a = 1/(I'+1)$. Thus a more correct solution for Goldstein's equation 17A will be obtained by setting $a = 1/(I'+1)$ when $t = 0$ in the integration of Eqn. (3). It follows that

$$a = \frac{1}{(S'+I')}\left\{S' + \left[\frac{I'(1-S')}{(I'+1)}\right]\exp\left[-\frac{(S'+I')\,t}{\dfrac{I'}{k_4+k_6} + \dfrac{S'}{k_2+k_5}}\right]\right\}. \qquad (6)$$

On plotting the theoretical curves calculated from the equation (see Fig. 2), it is again found that the experimental data obtained by Goldstein (1944) can be fitted just as well by an equation where the value of (k_4+k_6) is high enough so that $I'/(k_4+k_6)$ can be neglected, as by an equation in which $(k_4+k_6) = 0{\cdot}32$ min.$^{-1}$.

Using these corrected equations, it would seem that the best fit to all the data of both figures is given by a value of 0·020 min.$^{-1}$ for (k_2+k_5) (see Figs. 1 and 2). The experimental data do not allow any definite value to be assigned to (k_4+k_6), however, nor do they contradict the fact that (k_4+k_6) must be >3500 min.$^{-1}$.

Although this discussion has dealt with the reaction between enzyme and substrate, it is obvious that a similar consideration will apply in the case of an inhibitor which itself is susceptible to slow hydrolysis by the enzyme. Thus the reaction between pseudo-cholinesterase and eserine, for example, should be written in the form

$$E_f + I_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_5} E_f + \text{reaction products}.$$

Then, as before in the case of the substrate, the experimental value of K_I will represent $(k_2+k_5)/k_1$ rather than k_2/k_1, and the value 0·020 min.$^{-1}$ will represent (k_2+k_5) rather than 'k_2'.

By the methods outlined by Goldstein (1944) k_5 itself can be determined, but a definite value can be assigned only if the enzyme concentration is known. Using a zone A equation despite the assumption of $E' = 12{\cdot}8$, Goldstein has calculated that $k_5 = 0{\cdot}00182$ min.$^{-1}$ for the destruction of eserine in horse serum as given by the data of Ellis, Plachte & Straus (1943). From the data given in a previous article (Myers, 1952), it can be calculated that the concentration of pseudo-cholinesterase in the full horse serum used by Ellis *et al.* (1943) was probably $6{\cdot}2 \times 10^{-8}$ M. Using $K_I = 3 \times 10^{-8}$ M-eserine, then $E' = E/K_I = 2{\cdot}1$ and the value of k_5 would become 0·011 min.$^{-1}$. It is interesting that this value of k_5

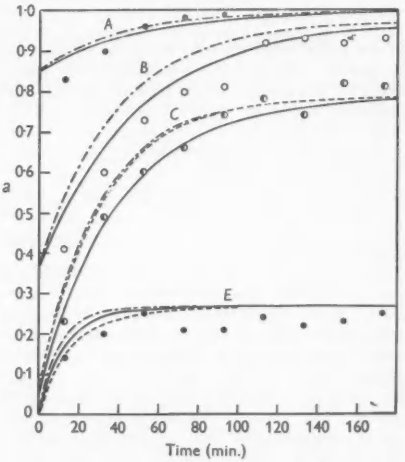

Fig. 2. Displacement of inhibitor by substrate. Acetylcholine added at zero time to an equilibrated mixture of dog serum and eserine. Data of fractional activity of enzyme (a) taken from table V of Goldstein (1944). Points near A with $5{\cdot}5 \times 10^{-9}$ M-, B with $5{\cdot}5 \times 10^{-8}$ M-, C with $5{\cdot}5 \times 10^{-7}$ M-, and E with $5{\cdot}5 \times 10^{-6}$ M-eserine. Theoretical curves calculated from equation 6, see text. – – –, $(k_2+k_5) = 0{\cdot}026$, $(k_4+k_6) = 0{\cdot}32$ min.$^{-1}$; –·–·–, $(k_2+k_5) = 0{\cdot}026$, $(k_4+k_6) > 3500$; these two lines are practically identical near points A and B; ——, $(k_2+k_5) = 0{\cdot}020$, $(k_4+k_6) > 3500$.

is of a similar order of magnitude to the value of (k_2+k_5) calculated previously, but this is only a very rough approximation since it is a value dependent on the results of three separate investigations. Further, the value of k_5 obtained with eserine and the pseudo-cholinesterase of horse serum cannot be strictly compared with the previous value of (k_2+k_5) obtained with eserine and the pseudo-cholinesterase of dog serum, even if the experimental conditions were not somewhat different in the three experiments.

EXPERIMENTAL AND RESULTS

The data given by Goldstein (1944) suffer from the disadvantage of being determined with dog serum using acetylcholine as substrate for pseudo-cholinesterase and eserine as cholinesterase inhibitor. Dog serum also contains appreciable

amounts of true cholinesterase (Mendel, Mundell & Rudney, 1943) which would also hydrolyse acetylcholine and would also be inhibited by the concentrations of eserine used to inhibit the pseudocholinesterase. In human serum, on the other hand, the amount of true cholinesterase is very small and would, in any case, have no effect upon the results when benzoylcholine is used as a specific substrate (Mendel *et al.* 1943) and the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)trimethylammonium bromide (Hoffman-La Roche, Nu 683) as a specific inhibitor (Hawkins & Gunter, 1946) for the pseudocholinesterase.

Table 1. *Destruction of Nu* 683 *by the pseudo-cholinesterase of human serum on incubation at* 37·5° *and* pH 7·4

(The solution contained 0·025 M-bicarbonate, a volume of dialysed human serum equivalent to 10% (v/v) native serum, and 2×10^{-8} M-Nu 683. After incubation for various periods of time, the activity of 5 ml. was measured using 0·006 M-benzoylcholine as substrate for the pseudocholinesterase. Three control serum solutions with no added inhibitor were treated similarly.)

Time of incubation (min.)	Activity in the first 10 min. after addition of substrate	Activity in the first 20 min. after addition of substrate
	Activity of the control solutions (μl. CO_2/5 ml. solution)	
15	163	330
340	157 av. 160	323 av. 326
510	159	325
	Activity of the serum solutions with Nu 683 calculated as a fraction (a) of the corresponding average control value	
15	0·054	0·072
30	0·041	0·052
60	0·043	0·059
120	0·029	0·051
200	0·060	0·075
260	0·094	0·101
320	0·157	0·168
380	0·321	0·324
410	0·421	0·419
430	0·502	0·509
450	0·533	0·549
480	0·679	0·674
510	0·748	0·753

To obtain comparable values for k_5 and (k_2+k_5), these reaction velocity constants were determined on the same enzyme-inhibitor system as was used previously for the determination of enzyme concentration (Myers, 1952), that is to say, with Nu 683 as inhibitor of the pseudo-cholinesterase activity of dialysed human serum.

The experimental methods used were essentially the same as those described in a previous article (Myers, 1952). A 0·025 M-bicarbonate buffer saturated with 5% CO_2 in 95% (v/v) N_2 gave a pH of 7·4 at 37·5°. The substrate in the first experiment below was 0·006 M-benzoylcholine; in the second experiment 0·06 M-acetylcholine was employed.

By following the change in the fractional activity a caused by enzymic destruction of inhibitor over a period of several hours, k_5 can be determined directly (see Goldstein, 1944; Ellis *et al.* 1943; Easson & Stedman, 1936). Under the conditions used, the most accurate estimate of k_5 could be obtained by using a concentration of inhibitor which caused almost complete inhibition initially (Fig. 3). However, it might be expected that the small activity initially observed during the first 2 hr. of the incubation would be more susceptible to errors caused by the displacement of combined inhibitor after the addition of substrate.

Theoretical curves for this type of displacement were shown in Fig. 2 above. But the results given in Table 1 show that it does not make much difference to these particular results whether the fractional activity is calculated for the first 10 min. or for the first 20 min. after addition of substrate. This does not mean that combined Nu 683 was not displaced by added benzoylcholine, but rather, that the displacement was very slow. Over longer periods of time after the addition of substrate, a marked displacement of combined inhibitor was evident at all stages of the experiment represented by Fig. 3. This confirms the previous conclusion that Nu 683 is a reversible competitive inhibitor of the pseudocholinesterase.

The values calculated from the activity observed in the first 10 min. after the addition of substrate were used as a somewhat better approximation to the 'inhibition' existing before substrate was added. On plotting these results and applying the correct theoretical equation

$$\left[\frac{1}{a_0}+\ln\frac{(1-a_0)}{a_0}+\mathrm{E}'\ln(1-a_0)\right] - \left[\frac{1}{a}+\ln\frac{(1-a)}{a}+\mathrm{E}'\ln(1-a)\right]=k_5\mathrm{E}'t \quad (7)$$

in place of Goldstein's equation 25, the experimental results are found to correspond fairly well with these theoretical curves (Fig. 3).

$$K_\mathrm{I}=8{\cdot}8\times10^{-10}\,\mathrm{M}\text{-Nu 683}$$

for this enzyme-inhibitor system and

$$\mathrm{E}=2{\cdot}3\times10^{-9}\,\mathrm{M}\text{-pseudo-cholinesterase}$$

in a solution of which 5 ml. give an activity of 100 μl. CO_2/20 min. with 0·006 M-benzoylcholine as substrate under the standard experimental conditions (Myers, 1952). For the above experiment, thus $\mathrm{E}=7{\cdot}5\times10^{-9}$ M-pseudo-cholinesterase and

$$\mathrm{E}'=\mathrm{E}/K_\mathrm{I}=8{\cdot}5.$$

Using this value for E′, the best average value of k_5 was 0·0119 min.$^{-1}$.

By measuring the rate of displacement of substrate by inhibitor, (k_2+k_5) was then determined. Under the conditions used, $\mathrm{E}'/\mathrm{S}'=0{\cdot}04$ so that zone

A equations can be applied. With other kinetic data from which (k_2+k_5) could be estimated (displacement of inhibitor by substrate, effect of dilution, combination of inhibitor with enzyme), the enzyme-inhibitor system would be in zone *B*, rendering the mathematical solution for (k_2+k_5) much more difficult.

In this second experiment, 0·06 M-acetylcholine was used as substrate for the pseudo-cholinesterase. The affinity of benzoylcholine for the pseudo-equivalent to $(a.k_6.E)$ (Goldstein, 1944). From the equation $S'=a/(1-a)$, $a=0·978$ when $S'=45$; from the data given previously (Myers, 1952), $E=1·72\times10^{-9}$ M-pseudo-cholinesterase at this particular concentration of serum. Substituting these values for a and E in the equation

$$(a.k_6.E)=9·76\times10^{-5}\text{ mole l.}^{-1}\text{ min.}^{-1},$$

then $$k_6=5·80\times10^{4}\text{ min.}^{-1}$$

and $$(k_4+k_6)\geqslant 5·80\times10^{4}\text{ min.}^{-1}.$$

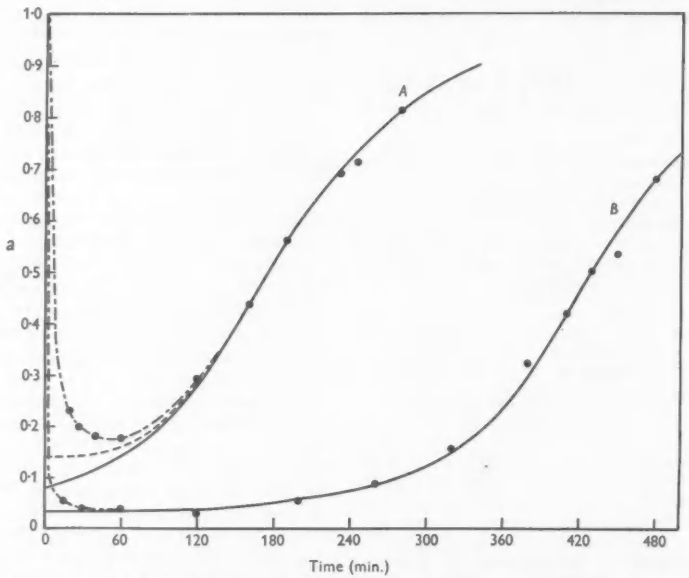

Fig. 3. Enzymic destruction of Nu 683 by the pseudo-cholinesterase of human serum. Experimental conditions as in Table 1. Ordinate fractional activity of enzyme (a). Theoretical curves (——) calculated from Eqn. (7) where $k_5=0·0119$ min.$^{-1}$, $E=7·5\times10^{-9}$ M-pseudo-cholinesterase. The experimental curve (–·–·–) deviates from the theoretical curve initially because of the fact that the inhibitor is added at zero time and requires 50–60 min. to come to equilibrium with the enzyme. Curve *A* using 1×10^{-6} M-Nu 683, curve *B* using 2×10^{-6} M-Nu 683.

cholinesterase of dialysed human serum is so high that an accurate determination of pS (0·5) (see Fig. 4) was impossible with the Warburg technique. Since an accurate value for S′ is necessary in determining (k_2+k_5), the use of benzoylcholine as substrate was not practicable for this experiment.

A plot of the activity at various substrate concentrations against the log of substrate concentration indicated that half-maximal activity is obtained with a concentration of $1·34\times10^{-3}$ M-acetylcholine (Fig. 4). Thus $K_s=1·34\times10^{-3}$ M and, for S = 0·06 M-acetylcholine, $S'=S/K_s=45$.

At this particular concentration of acetylcholine, the activity observed with the 2% (v/v) serum solution used was 218 µl. CO_2/5 ml. solution/20 min. or 0·0976 m-mole/l./min. This rate of hydrolysis is

In the theoretical equation for change in a as the substrate is displaced by added inhibitor, these reaction constants occur in the term

$$\left[\frac{I'}{(k_4+k_6)}+\frac{S'}{(k_2+k_5)}\right].$$

When $I'=228$, as in the present experiment, $I'/(k_4+k_6)=0·004$ min. For $S'=45$ and

$$(k_2+k_5)=0·0119\text{ min.}^{-1},$$

on the other hand, $S'/(k_2+k_5)=3800$ min. Therefore, under these experimental conditions, $I'/(k_4+k_6)$ will be quite negligible in comparison to $S'/(k_2+k_5)$. Thus Eqn. (4) can be rewritten in the simplified form

$$a=\frac{1}{(S'+I')}\{S'+I'.\exp[-t(k_2+k_5)\ (S'+I')/S']\}. \quad (8)$$

Using this equation, the best average value of (k_2+k_5) can be calculated for the experimental points of Fig. 5. Satisfactory agreement with the experimental points is obtained by giving (k_2+k_5)

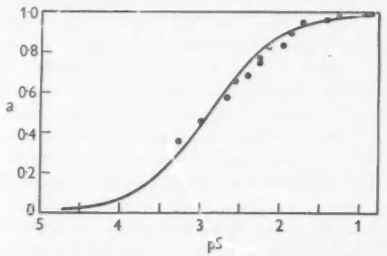

Fig. 4. Pseudo-cholinesterase activity at various concentrations of acetylcholine. Ordinate: fractional activity of enzyme (a). Abscissa: negative log. of the molar concentration of acetylcholine. Theoretical curve calculated from the equation $S=K_s a/(1-a)$ using the value $K_s=1{\cdot}34\times10^{-3}$ M.

a value of 0·0119 min.$^{-1}$. The experimental error precludes the determination of a more precise value for these velocity constants; but it can be seen from Fig. 5 that the rate of displacement of substrate by inhibitor is, if anything, slightly slower than the rate expected if $(k_2+k_5)=0{\cdot}0119$ min.$^{-1}$. This would mean that (k_2+k_5), as calculated from this experiment, might be slightly less than 0·0119 min.$^{-1}$. Since this work was completed, a method has been described for the determination of the limiting concentration of pseudo-cholinesterase in serum by the use of an irreversible fluorophosphonate inhibitor (Berry, 1951). Therefore the pseudo-cholinesterase concentration in human serum was estimated by a similar technique using di*iso*propyl fluorophosphonate as enzyme inhibitor in the present investigation. The results indicated that the enzyme concentration was $2{\cdot}20\times10^{-9}$ M-pseudo-cholinesterase (range $2{\cdot}12$–$2{\cdot}34\times10^{-9}$ M)/(100 μl. CO_2/20 min.) activity under the standard conditions used above. This would mean that the value of k_5 is about 0·0115 min.$^{-1}$, which gives an even better fit to the experimental points of Fig. 5.

From these results it can be concluded that (k_2+k_5) is not significantly greater than k_5 alone, in other words, that k_2 must be of negligible value in comparison to k_5. This would mean that in this particular case the 'reversible reaction' between enzyme and inhibitor can, for practical purposes, be written as

$$E+I \rightarrow EI \rightarrow E+\text{reaction products},$$

and that the Michaelis constant K_1 could be equated to k_5/k_2.

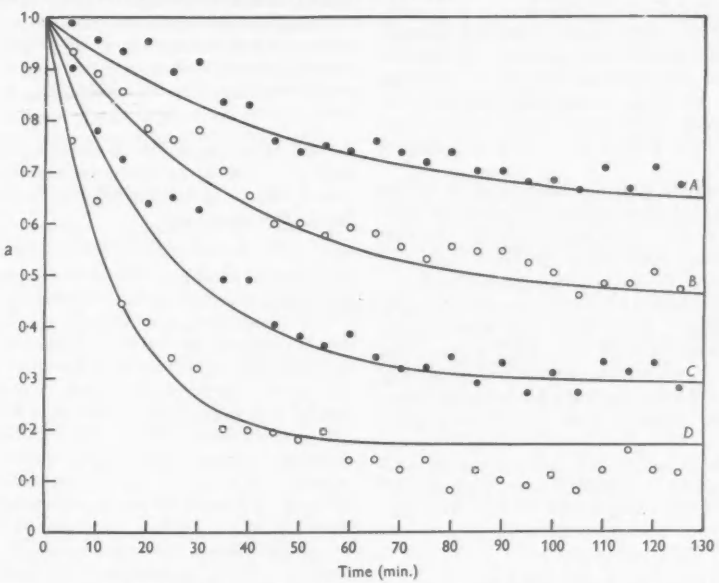

Fig. 5. Displacement of substrate by inhibitor. Nu 683 was added at zero time to an equilibrated mixture containing 2% (v/v) dialysed human serum and 0·06 M-acetylcholine. Ordinate: fractional activity of enzyme (a). Theoretical curves were calculated from Eqn. (8) (see text) for $S'=45$ and $(k_2+k_5)=0{\cdot}0119$ min.$^{-1}$; $I'=28{\cdot}5$ for curve A, 56·9 for curve B, 114 for curve C, and 228 for curve D.

DISCUSSION

The determination of the individual rate constants for the reactions involved in the inhibition of pseudo-cholinesterase by Nu 683 showed that the rate of dissociation of combined enzyme-inhibitor (k_2) was negligible in comparison to the rate of hydrolysis of combined inhibitor (k_5). The rate of hydrolysis of a substrate such as acetylcholine is much too high to allow the direct determination of the corresponding velocity constants k_4 and k_6. However, it seems quite possible that k_4 may also have a negligible value in comparison to the very high value of k_6. No proof of this assumption can be given as yet, but for practical purposes we could consider that the Michaelis constant $K_s = (k_4 + k_6)/k_3 \geqslant k_6/k_3$.

In the case of the hydrolysis of acetylcholine by pseudo-cholinesterase, k_6 was found to have a value of $5{\cdot}8 \times 10^4$ min.$^{-1}$, while $K_s = 1{\cdot}34 \times 10^{-3}$ moles/l. under the experimental conditions. Thus

$$k_3 \geqslant k_6/K_s = 4{\cdot}3 \times 10^7 \text{ l. mole}^{-1} \text{ min.}^{-1}.$$

The corresponding constant for Nu 683,

$$k_1 = k_5/K_1 = 1{\cdot}35 \times 10^7 \text{ l. mole}^{-1} \text{ min.}^{-1}.$$

It appears that the rate of combination with pseudo-cholinesterase differs only by about three times for acetylcholine and for Nu 683. On the other hand, the rate of hydrolysis of acetylcholine

$$(k_6 = 5{\cdot}8 \times 10^4 \text{ min.}^{-1})$$

would be nearly 5×10^6 times greater than the rate of hydrolysis of Nu 683 ($k_5 = 0{\cdot}0119$ min.$^{-1}$) by pseudo-cholinesterase. Apparently the chief difference between the reactions of acetylcholine and of Nu 683 with pseudo-cholinesterase lies in the rate of hydrolysis of these two compounds by the pseudo-cholinesterase.

For comparative purposes, approximate values of the corresponding constants (k_3 and k_6) were calculated from data on the true cholinesterase concentration which have been obtained with the *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250) as inhibitor (Myers, 1952). These indicated that an enzyme concentration of $6{\cdot}3 \times 10^{-10}$ moles true cholinesterase/l. will give an activity of 100 μl. CO_2/20 min./5 ml. solution, or $4{\cdot}46 \times 10^{-5}$ moles/l./min., at a substrate concentration of 0·03 M-acetyl-β-methylcholine. From results obtained previously (Myers, 1951), we can calculate that S′ has an average value of about 37 with 0·02 M-acetyl-β-methylcholine and therefore $K_s = 5{\cdot}4 \times 10^{-4}$ M. At a concentration of 0·03 M-acetyl-β-methylcholine, S′ = 56 and $a = 0{\cdot}982$. Then, as before,

$$k_6 = (\mathrm{dS}/\mathrm{d}t)/a\mathrm{E} = 7{\cdot}2 \times 10^4 \text{ min.}^{-1}$$

and $$k_3 \geqslant k_6/K_s = 1{\cdot}3 \times 10^8 \text{ l. mole}^{-1} \text{ min.}^{-1}.$$

These values are slightly higher (1·2 and 3·0 times respectively) than the values of the same reaction constants for the hydrolysis of acetylcholine by the pseudo-cholinesterase of human serum.

The most striking thing about all of these rate constants is the large order of magnitude, indicating a very high activity of the cholinesterases towards acetylcholine and related substrates. In the hydrogen peroxide-peroxidase system used by Chance (1943), k_3 also had a very high value which was, in fact, of the same order of magnitude as those obtained above with the cholinesterases. The value given for k_3 was $1{\cdot}2 \times 10^7$ l. mole^{-1} sec.$^{-1}$ or $7{\cdot}2 \times 10^8$ l. mole^{-1} min.$^{-1}$, as compared with the values $1{\cdot}35 \times 10^7$ to $1{\cdot}3 \times 10^8$ l. mole^{-1} min.$^{-1}$ calculated above. On the other hand, the destruction constant, k_6, varied from nearly zero to higher than 300 min.$^{-1}$, depending upon the concentration of a third reactant which reacted with the activated enzyme-substrate complex (Chance, 1943). In the case of the cholinesterases, the third reactant is probably water, the concentration of which is very high and constant under the experimental conditions.

In the particular case investigated, the reaction between the reversible inhibitor and enzyme could be represented best by an equation of the form

$$\mathrm{E} + \mathrm{I} \rightarrow \mathrm{EI} \rightarrow \mathrm{E} + \text{reaction products.}$$

Obviously this conclusion cannot be extended to all reversible competitive inhibitors of cholinesterases. A simple cationic inhibitor such as the sodium ion or choline is not susceptible to hydrolysis by cholinesterase and in such a case the reversible reaction will be strictly of the classical form

$$\mathrm{E} + \mathrm{I} \rightleftharpoons \mathrm{EI}.$$

In still other cases, the reaction between enzyme and a reversible competitive inhibitor which is susceptible to hydrolysis may be represented best by the full equation

$$\mathrm{E} + \mathrm{I} \rightleftharpoons \mathrm{EI} \rightarrow \mathrm{E} + \text{reaction products,}$$

but no good examples of this third type of reaction have been described in the literature as yet.

The assumption, that the enzymic reaction with either substrate or inhibitor may follow the first type of equation, will not invalidate the basic theory of enzyme kinetics as developed for reactions of the second and third type (Michaelis & Menten, 1913; Briggs & Haldane, 1925; Goldstein, 1944). The main difference will be in the velocity constants which are represented in the Michaelis constants K_1 and K_s. It does of course lay more emphasis on the destruction of inhibitor or substrate by the enzyme, but this is a factor which must always be taken into account when measuring the initial constant velocity of the enzyme activity.

As noted above, the main difference between acetylcholine and Nu 683 in their reactions with pseudo-cholinesterase would appear to be the large

difference in the relative rates of hydrolysis. This forms an interesting comparison with the data of Chance (1943) on peroxidase, in which case the Michaelis constant K_s, with the same substrate throughout, was directly proportional to the velocity constant k_6 for the rate of destruction of the substrate. The apparent dependence of K_s upon k_6 would suggest that the values of k_1 and k_3 may be a measure of physical factors limiting the rate of combination of enzyme with substrate or inhibitor. However, it is rather improbable that this comparison between the values of k_6 and K_s could be extended to all enzymes substrates under all experimental conditions.

The effect of the experimental conditions upon the values obtained for these velocity constants remains to be investigated. It has been demonstrated with cholinesterase that the Michaelis constants for both substrate and inhibitor may be considerably affected by the presence of salts in the medium (Mendel & Rudney, 1945; Myers, 1950). Thus the salt concentration must have a significant effect upon the observed values of the individual reaction constants. However, it would seem logical that these rate constants should be determined in a medium containing as few interfering factors as possible; the inherent velocity constants of the enzyme-inhibitor or enzyme-substrate system are probably those which would be obtained in the complete absence of salts or other interfering factors in the medium. Since high concentrations of salts are not necessary for the activity of the cholinesterases, the best practical solution to this problem is probably obtained by using only the low concentration of buffer solution required to maintain pH.

SUMMARY

1. The theoretical formulations of Goldstein (1944) were based on the assumption that the reaction between an enzyme (E) and its substrate (S) could be adequately represented by an equation of the form

$$E + S \rightleftharpoons ES.$$

These formulations have been modified to comply with the correct equation for the overall reaction

$$E + S \rightleftharpoons ES \rightarrow E + \text{reaction products.}$$

2. The velocity constants for the reactions between the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)trimethylammonium bromide (Nu 683) and the pseudo-cholinesterase of human serum have been determined by the use of these modified theoretical equations. The results obtained were as follows: Michaelis constant $K_I = (k_2 + k_5)/k_1 = 8{\cdot}8 \times 10^{-10}$ mole l.$^{-1}$; $k_5 = 0{\cdot}0119$ min.$^{-1}$ (rate of hydrolysis of inhibitor I); $(k_2 + k_5) = 0{\cdot}0119$ min.$^{-1}$; k_2 = negligible value (rate of dissociation of EI); $k_1 = 1{\cdot}35 \times 10^7$ l. mole^{-1} min.$^{-1}$ (rate of combination of E with I).

3. The corresponding velocity constants between acetylcholine and the pseudo-cholinesterase were: Michaelis constant $K_s = (k_4 + k_6)/k_3 = 1{\cdot}34 \times 10^{-3}$ mole l.$^{-1}$; $k_6 = 5{\cdot}8 \times 10^4$ min.$^{-1}$ (rate of hydrolysis of S); $k_3 \geqq 4{\cdot}3 \times 10^7$ l. mole^{-1} min.$^{-1}$ (rate of combination of E with S).

4. The significance of these results has been discussed. In the particular case of Nu 683 as inhibitor of the pseudo-cholinesterase activity of dialysed human serum, it would appear that the 'reversible reaction' between the enzyme and inhibitor could for practical purposes be represented by an equation of the form

$$E + I \rightarrow EI \rightarrow E + \text{reaction products.}$$

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REFERENCES

Berry, W. K. (1951). *Biochem. J.* **49**, 615.
Briggs, G. E. & Haldane, J. B. S. (1925). *Biochem. J.* **19**, 338.
Chance, B. (1943). *J. biol. Chem.* **151**, 553.
Easson, L. H. & Stedman, E. (1936). *Proc. roy. Soc.* B, **121**, 142.
Ellis, F., Plachte, F. L. & Straus, O. H. (1943). *J. Pharmacol.* **79**, 295.
Goldstein, A. (1944). *J. gen. Physiol.* **27**, 529.
Hawkins, R. D. & Gunter, J. M. (1946). *Biochem. J.* **40**, 192.
Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
Mendel, B. & Rudney, H. (1945). *Science*, **102**, 616.
Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.
Myers, D. K. (1950). *Arch. Biochem.* **27**, 341.
Myers, D. K. (1951). *Arch. Biochem. Biophys.* **31**, 29.
Myers, D. K. (1952). *Biochem. J.* **51**, 303.
Straus, O. H. & Goldstein, A. (1943). *J. gen. Physiol.* **26**, 559.

Volatile Acids in the Secretion Products (Hair Grease) of the Skin

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The secretory products of the sweat glands and the sebaceous glands are in several respects physiologically important. The function of the sweat in the excretion of waste products and in the regulation of body temperature is well known. The sebum from the sebaceous glands doubtless aids in preventing the penetration of harmful substances into the skin, it affords protection against the disintegrating influence of external moisture, it gives pliability to the skin, and it contains provitamin D (Dukes, 1947). In healthy animals it confers on the hair its glossy appearance, whilst in illness the fur may be dry and the hair brittle perhaps due to lack of sebum. Moreover, the secretion products of the skin are said to have antimicrobial properties. Finally, the odorous substances are of considerable importance in the relations between male and female animals and between mother and young.

Very little is known with certainty about the odorous substances in the skin secretions. Nevertheless, it is usually assumed that the repugnant smell of male goats originates from skin secretions and is due to caproic and caprylic acid (Zwaardemaker, 1895). However, in scrutinizing the literature, we did not find reliable chemical analyses corroborating this assumption. It seemed, therefore, worth while to determine the volatile acids in extracts of hair of male goats in comparison with other animals with the aid of the newer methods of chromatography. We found, however, that the C_6, C_8 and C_{10} acids were by no means abundant. Nevertheless, the volatile acids in the hair extracts of different species of animals presented several interesting features. Moreover, the study of the skin secretions, as compared with the milk secretion, is of fundamental importance, because the udder can be considered to be a huge complex of modified skin glands.

METHODS

The chromatographic method used was the one initiated by Ramsey & Patterson (1948) as improved upon by Nijkamp (1951). As far as the extraction of the hair is concerned, the following procedure was adopted. The hair was collected from the back and sides of the animals and stored at $-20°$ if not used immediately. After clipping and, if necessary, drying over blue silica gel, about 20 g. of hair was extracted for several hours with peroxide-free diethyl ether. The ether was washed with a dilute aqueous solution of K_2CO_3. After evaporation of the ether, a residue of fatty material was left behind and has been named by us the 'lipid fraction'. It includes the triglycerides, the waxes, etc. but not, of course, the free fatty acids. After saponification with a solution of NaOH in acid-free ethanol, the volatile acids were collected by steam distillation and separated on a silica gel column as described (Nijkamp, 1951).

The watery layer separated from the lipid fraction will be termed the 'K_2CO_3 fraction'. It also contained some volatile acid and was therefore treated separately. After evaporation to small volume and acidification, the volatile acids were collected by steam distillation and separated on a silica gel column.

Finally, the hair, as left after the ether extraction, was (at 35–40°) extracted once more, this time with a dilute aqueous solution of $KHCO_3$ containing enough K_2CO_3 so that the reaction remained faintly alkaline to phenolphthalein after the solution had been added to the hair. The extract plus washings was called the 'alkaline hair extract'. It was evaporated down to small volume and treated in the same way as the watery layer separated from the ether extract mentioned above.

All processes were shown by special experiments to be such that no breakdown of glycerides or waxes could occur during the extractions. The volatile acids obtained after saponification of the lipid fraction therefore originated from glycerides, and perhaps waxes and allied compounds. On the other hand, the volatile acids in the K_2CO_3 fraction and in the alkaline hair extract must have been present in the hair as such or in soaps; minor quantities may also have been produced in these watery extracts during evaporation and steam distillation as a result of decomposition of non-lipid material present in small amounts. There would, perhaps, have been some reason to combine the volatile acids from these two watery extracts. Nevertheless, in Table 1 they have been kept apart. Presumably, most of the free acids are found in the K_2CO_3 fraction and most of the soaps in the alkaline hair extract.

Although it was our intention to study in the first place the acids C_4–C_{10}, the figures for C_2 and C_3 are also given. The latter two acids must, however, be considered as minimum values because C_2 and C_3 acids are taken up by the solvent (*iso*-octane) with more difficulty than the acids C_4–C_{10}.

In Table 1, data are set out on C_2, C_3, C_4, C_5, C_6, C_8 and C_{10} acids. In some instances, however, still other volatile acids must have been present, presumably C_7 and C_9 acids and an unknown volatile acid moving on the column between C_3 and C_4.

RESULTS

Lipid fraction

Although precautions had been taken to prevent any contamination whatever with acetic and propionic acids, many samples (especially those from ruminants) did contain measurable quantities of those compounds. It must, therefore, be concluded that the two acids were present (as esters) in the lipid fraction. The biggest quantity was only about 0·1 %, but this represents a minimum. As a matter of fact, French, Hunter, Martin & Popják (1951) have recently isolated and identified acetic acid from goat milk fat. In addition, traces of propionic acid and valeric acid have also been identified by these authors.

The proportions of the volatile acids in the various lipid fractions presented interesting features. In the cases of the horse, pig, rabbit, cat, guinea pig, rat and man all volatile acids up to C_8 were only present in minor or even negligible quantities. In most instances, however, the quantity of C_{10} acid was somewhat higher. Up to now only one sample from each of these species has been examined.

In the lipid fraction of three samples of goat hair all members (C_2, C_3, C_4, C_5, C_6, C_8, C_{10} acids) were present in appreciable quantities, the aggregate amounting to about 3 % of the lipid fraction. This great variety of volatile acids seems characteristic of ruminants, for it was also found with hair of cattle and of sheep. Nevertheless, with the latter two species, the results differed somewhat from those associated with goats. With cows (either in milk or not) a shift was shown to C_8 and C_{10} acids. The quantities of C_2, C_3, C_4 and C_5 acids were smaller, the quantities of C_6 acid about equal and of C_8 and C_{10} acids larger than with male goats. With the bull, the distribution of the acids was about the same as with cows. Thus, with male goats, contrary to what was anticipated, the quantities of C_6, C_8 and C_{10} were by no means larger than with cattle.

With sheep, the distribution of the volatile acids was different again, with C_4 and C_5 acids predominating. The other volatile acids, though not absent, were present in so much smaller quantities that the aggregate of volatile acids amounted to only half the quantity found in the lipid fractions of goats and cattle.

Still another type of distribution prevailed with the dogs. The total quantity of volatile acids amounted to about 5 %, and with five animals consisted almost exclusively of valeric acid. With three other dogs, some other volatile acids were also present, but only in small quantities, most of them being C_6, C_8 and C_{10} acids. Whether the C_5 is *n*-valeric acid or *iso*valeric acid has not yet been established. It is of interest that *iso*valeric acid has been recognized as an important constituent of the fat of the dolphin and the porpoise. The percentages (by weight in the fatty acids) in the depot fat of the body, head and jaw of the porpoise (*Phocaena communis*) were respectively 13·6, 20·8 and 25·3 (Lovern, 1934).

K_2CO_3 *fraction and alkaline hair extract*

As a rule, the total quantities of volatile acids in these fractions were not very different. Except with the sheep, they amounted to only a few mg./100 g. of hair. Nevertheless, with the horse, pig, rabbit, guinea pig, rat and man, the totals were somewhat higher than those in the lipid fractions, which were almost devoid of volatile acids. With the cat they were a little lower. The question whether the small quantities of volatile acids in the K_2CO_3 fraction and in the alkaline hair extract have been secreted as such or as esters or whether they might partly be decomposition products, cannot be settled from our experiments. In most cases C_2 and C_3 acids were preponderant. In many instances, however, the amount of C_{10} acid was also somewhat higher than that of the intermediate volatile acids.

Nearly the same result (C_4, C_5, C_6 low, and C_2, C_3, C_{10} as a rule somewhat higher) was arrived at with the cows and the goats. Again with the male goats, C_6, C_8 and C_{10} acids were by no means higher than with the cows and the bull. On the contrary, with the bull, C_6 was distinctly higher. Thus, with the goats, neither the lipid fraction nor the other fractions showed high contents of C_6, C_8 and C_{10} acids. Still it was quite striking that these fractions often displayed a strongly offensive goat smell. For this reason, we are inclined to believe that the smell should not be attributed to C_6–C_{10} acids but rather to other, still unknown, odorous substances.

With the dogs the quantity of C_5 acid was again conspicuously higher than with the other species of animals. With the dogs it was only second to C_2 acid.

With the sheep, the volatile acids in the two fractions under consideration were distinctly larger than with the other animals, the totals being in two instances as big as in the lipid fraction. The amounts of the individual acids varied considerably. The averages of the individual acids, however (including C_2), did not show big variations. More than with the other species of animals we got the impression that, with sheep, some decomposition of the esters had taken place. The fact that the wool protects the grease from being continuously rubbed off by the action of the animal may contribute to this phenomenon.

SUMMARY

1. The volatile fatty acids C_2, C_3, C_4, C_5, C_6, C_8 and C_{10} (free and as esters) of the skin secretions (hair grease) of man and of several species of animals have been studied. The quantities of these acids

Table 1. *Distribution of volatile fatty acids in various fractions obtained from hair grease of various species of animals*

	Exp.	Lipid fraction of hair (g./100 g.)	Lipid fraction: Volatile acids (g./100 g. of lipid fraction) C_2	C_3	C_4	C_5	C_6	C_8	C_{10}	Total	Lipid fraction: Volatile acids (mg. in the lipid fraction of 100 g. of hair) C_2	C_3	C_4	C_5	C_6	C_8	C_{10}	Total
Goat (M.)	3	2·14	0·08	0·04	0·26	0·14	0·16	0·80	2·32	**3·80**	1·8	0·9	5·4	3·0	3·4	17·0	49·6	**81·1**
	23	1·37	0·12	0·06	0·61	0·24	0·12	0·68	1·28	**3·11**	1·6	0·8	8·4	3·4	1·7	9·4	17·6	**42·9**
	24	1·98	0·08	0·07	1·41	0·57	0·03	0·09	0·24	**2·49**	1·6	1·4	28·0	11·3	0·5	1·8	4·8	**49·4**
Cow D, no milk	0	2·30	0·03	0·02	0·06	0·00	0·12	0·56	2·44	**3·23**	0·8	0·4	1·5	0·0	2·8	12·8	56·0	**74·3**
Heifer, no milk	6	4·33	0·04	0·03	0·03	0·00	0·08	1·06	1·29	**2·53**	1·6	1·2	1·4	0·0	3·4	46·1	55·9	**109·6**
Cow, 8 kg. milk	1	7·45	0·02	0·02	0·03	0·01	0·10	0·86	1·64	**2·68**	1·2	1·2	2·2	1·0	7·4	63·9	122·4	**199·3**
Cow, 6 kg. milk	2	7·03	0·01	0·01	0·02	0·00	0·09	1·18	1·87	**3·18**	0·9	0·7	1·4	0·0	6·4	83·1	131·3	**223·8**
Cow, 13 kg. milk	5	9·07	0·04	0·04	0·04	0·00	0·11	1·25	1·63	**3·11**	3·5	3·6	4·0	0·0	10·4	113·7	148·1	**283·3**
Bull	4	5·52	0·09	0·03	0·01	0·00	0·09	1·02	1·68	**2·92**	5·0	1·8	0·4	0·0	4·8	56·2	92·6	**160·8**
Sheep (F.)	26	6·16	0·01	0·01	0·04	0·27	tr.	0·04	0·11	**0·48**	0·8	0·5	2·3	16·4	0·2	2·4	6·9	**29·5**
	27	3·97	0·03	0·00	0·39	0·74	0·07	0·02	0·09	**1·34**	1·4	0·0	15·6	29·4	2·9	0·8	3·6	**53·7**
	28	9·47	0·05	0·02	0·48	0·69	0·01	0·00	0·28	**1·53**	4·3	1·6	45·4	65·2	1·2	0·0	26·6	**144·3**
Fat-tailed sheep*	22	7·57	tr.	tr.	0·57	0·79	0·01	0·04	0·52	**1·93**	tr.	0·4	43·5	60·0	1·0	3·2	39·4	**147·5**
Horse (F.)	12	2·70	tr.	0·01	0·00	0·00	0·00	0·03	0·04	**0·08**	0·1	0·3	0·0	0·0	0·0	0·9	1·2	**2·5**
Pigs (F. and M.)	10	0·22	0·02	0·02	0·07	0·02	0·00	0·09	0·09	**0·31**	tr.	tr.	0·2	0·1	0·0	0·2	0·2	**0·7**
Rabbit (M.)	11	0·64	0·04	0·00	0·00	0·28	0·00	0·00	0·00	**0·32**	0·2	0·0	0·0	1·8	0·0	0·0	0·0	**2·0**
Dog (M.)	8	4·26	0·00	0·00	0·00	6·63	0·00	0·00	0·00	**6·63**	0·0	0·0	0·0	282·2	0·0	0·0	0·0	**282·2**
	13	2·59	0·14	0·00	0·00	4·37	0·00	0·00	0·00	**4·51**	3·6	0·0	0·0	112·9	0·0	0·0	0·0	**116·5**
	16	2·63	0·06	0·00	0·00	3·79	0·07	0·05	0·20	**4·17**	1·5	0·0	0·0	99·7	1·8	1·4	5·4	**109·8**
	17	2·99	0·00	0·00	0·00	4·66	0·00	0·00	0·00	**4·66**	0·0	0·0	0·0	139·2	0·0	0·0	0·0	**139·2**
	18	3·04	0·00	0·00	0·00	5·22	0·00	0·00	0·00	**5·22**	0·0	0·0	0·0	158·4	0·0	0·0	0·0	**158·4**
	19	3·55	tr.	0·00	0·74	4·10	0·22	0·16	0·19	**5·41**	tr.	0·0	26·4	145·6	7·8	5·8	6·9	**192·5**
	20	4·38	0·00	0·00	0·00	6·60	0·00	0·00	0·00	**6·60**	0·0	0·0	0·0	288·8	0·0	0·0	0·0	**288·8**
	21	1·67	tr.	0·13	0·00	4·49	0·00	0·19	0·28	**5·09**	tr.	2·2	0·0	74·9	0·0	3·2	4·6	**84·9**
Cat (M.)	15	1·60	0·04	tr.	0·01	tr.	0·02	0·07	0·15	**0·29**	0·7	0·1	0·1	tr.	0·2	1·2	2·4	**4·7**
Guinea pig (M.)	7	1·22	0·03	0·04	0·00	0·00	0·00	0·04	0·06	**0·17**	0·4	0·4	0·0	0·0	0·0	0·5	0·7	**2·0**
Rats (F. and M.)	25	3·15	tr.	tr.	0·01	0·01	tr.	0·01	0·00	**0·03**	0·2	0·1	0·4	0·3	0·1	0·3	0·0	**1·4**
Man† (M.)	14	1·74	0·04	tr.	0·01	tr.	0·00	0·03	0·05	**0·13**	0·8	tr.	0·2	0·1	0·0	0·6	0·8	**2·5**

	Exp.	Lipid fraction of hair (g./100 g.)	K_2CO_3 fraction: Volatile acids (mg./100 g. of hair) C_2	C_3	C_4	C_5	C_6	C_8	C_{10}	Total	Alkaline hair extract: Volatile acids (mg./100 g. of hair) C_2	C_3	C_4	C_5	C_6	C_8	C_{10}	Total
Goat (M.)	3	2·14	1·6	0·0	2·8	0·0	0·0	0·4	1·3	**6·1**	6·6	1·6	0·0	0·0	0·0	0·0	0·0	**8·2**
	23	1·37	0·6	0·0	0·0	0·1	0·3	0·4	1·2	**2·6**	7·6	0·0	5·3	0·2	0·6	0·4	2·4	**16·5**
	24	1·98	2·5	0·8	0·6	0·4	0·7	0·8	1·2	**7·0**	4·3	0·5	1·2	0·0	0·6	0·6	1·0	**8·2**
Cow D, no milk	0	2·30	3·0	1·5	0·2	0·0	0·4	0·6	2·4	**8·1**	0·9	1·1	0·0	0·0	0·0	0·0	0·0	**2·0**
Heifer, no milk	6	4·33	0·2	0·4	0·0	0·0	0·2	0·4	1·2	**2·4**	4·0	0·8	0·0	0·0	0·0	0·4	1·3	**6·5**
Cow, 8 kg. milk	1	7·45	1·0	0·6	0·3	0·0	0·2	0·2	1·0	**3·3**	1·2	0·2	0·0	0·0	0·0	0·0	0·0	**1·4**
Cow, 6 kg. milk	2	7·03	2·0	0·8	0·0	0·0	0·4	0·2	0·7	**4·1**	0·8	0·2	0·0	0·0	0·0	0·0	0·0	**1·0**
Cow 13 kg. milk	5	9·07	3·2	?	0·0	0·0	1·7	0·4	1·7	**7·0**	2·2	0·9	0·0	0·0	0·0	0·0	0·0	**3·1**
Bull	4	5·52	8·9	11·8	>82·0	4·5	13·2	1·6	3·1	**>125·1**	3·8	1·7	17·7	0·7	3·8	0·0	0·0	**27·7**
Sheep (F.)	26	6·16	1·4	2·4	3·8	1·6	0·0	2·0	2·8	**14·0**	8·0	4·8	0·4	1·0	0·2	0·6	0·8	**15·8**
	27	3·97	1·8	3·5	1·6	5·3	1·4	2·5	0·6	**16·7**	15·0	11·7	3·1	0·7	0·0	0·8	0·0	**31·3**
	28	9·47	2·0	0·1	0·8	0·5	1·3	0·6	0·4	**5·7**	8·0	0·0	19·4	2·4	1·0	0·4	1·0	**32·2**
Fat-tailed sheep*	22	7·57	2·4	0·0	1·6	2·6	1·6	0·8	1·6	**10·6**	2·4	0·0	1·2	2·2	0·4	0·5	0·4	**7·1**
Horse (F.)	12	2·70	2·4	0·0	0·7	0·2	0·1	0·4	±0·6	**±4·4**	2·2	0·2	0·4	0·0	0·0	±0·3	±0·4	**±3·5**
Pigs (F. and M.)	10	0·22	2·2	0·5	0·1	0·3	0·2	0·4	0·3	**4·0**	1·8	0·8	0·4	0·4	tr.?	0·0	0·0	**3·4**
Rabbit (M.)	11	0·64	3·0	0·4	0·2	0·1	tr.	0·4	0·2	**4·3**	4·3	tr.	0·0	0·2	0·0	0·0	0·0	**4·5**
Dog (M.)	8	4·26	1·3	0·2	0·0	0·8	0·0	0·0	0·0	**2·3**	1·8	0·4	0·0	1·2	0·0	0·0	0·0	**3·4**
	13	2·59	1·6	0·4	0·3	0·4	0·0	1·1	1·4	**5·2**	1·6	0·2	0·0	1·4	0·0	0·0	0·0	**3·2**
	16	2·63	1·1	0·4	0·6	0·2	1·8	2·2	10·2	**16·5**	1·4	0·3	0·0	1·0	0·0	0·0	0·0	**2·7**
	17	2·99	0·0	0·0	0·0	0·2	0·3	0·4	1·9	**2·8**	0·4	tr.	0·0	0·8	0·0	0·0	0·0	**1·2**
	18	3·04				Not determined								Not determined				
	19	3·55				Not determined								Not determined				
	20	4·38				Not determined								Not determined				
	21	1·67				Not determined								Not determined				
Cat (M.)	15	1·60	1·0	0·2	tr.	0·1	0·1	0·4	0·9	**2·7**	1·0	0·0	0·0	0·0	0·0	0·0	0·8	**1·8**
Guinea pig (M.)	7	1·22	3·4	3·6	0·0	0·0	0·0	0·0	0·0	**7·0**	1·8	2·6	0·0	0·0	0·0	0·0	0·0	**4·4**
Rats (F. and M.)	25	3·15	1·6	0·9	1·0	0·8	1·0	0·7	0·7	**6·7**	2·6	1·1	1·1	0·5	0·4	0·1	0·0	**5·8**
Man† (M.)	14	1·74	1·3	1·2	0·1	0·4	0·0	3·2	6·1	**12·3**	2·9	0·0	0·0	0·0	0·0	0·0	0·0	**2·9**

* Only hair from the head.
† Hair from the head; the hair was not washed during the period of gathering.
tr. = trace.

found in the secretions of man, horse, rabbit, cat, guinea pig and rat were very small.

2. With male goats, cattle, sheep and dogs, appreciable quantities were found, mainly in the lipid fraction of the hair, i.e. the ether extract from which the free acids had been removed. The acids (including some C_2 and C_3 acids), were therefore present in this fraction in combined form. The aggregates of volatile fatty acids C_2–C_{10} in the lipid fractions amounted for the four species of animals mentioned respectively to about 3, 3, 1·5 and 5 %.

3. In the lipid fractions relating to the three species of ruminants all seven volatile acids were present. However in cattle, C_8 and C_{10} acids were preponderant, whilst in sheep C_4 and C_5 acids predominated. In male goats the seven volatile acids were more evenly distributed, but C_8 and C_{10} acids predominated. The lipid fractions from eight dogs contained virtually C_5 acid only.

5. It is questionable whether the offensive smell of male goats is really due to C_6 and C_8 as is generally assumed.

REFERENCES

Dukes, H. H. (1947). *The Physiology of Domestic Animals*, p. 427.

French, T. H., Hunter, G. D., Martin, A. J. P. & Popják, G. (1951). *Biochem. J.* **48**, vi.

Lovern, J. A. (1934). *Biochem. J.* **28**, 394.

Nijkamp, H. J. (1951). *Analytica Chimica Acta*, **5**, 325.

Ramsey, L. L. & Patterson, W. J. (1948). *J. Ass. off. agric. Chem., Wash.*, **31**, 139.

Zwaardemaker, H. (1895). *Die Physiologie des Geruchs.*

The Occurrence of Growth Factors for *Lactobacillus leichmannii*, *Streptococcus faecalis* and *Leuconostoc citrovorum* in the Tissues of Pernicious Anaemia Patients and Controls

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The chief therapeutic substances that have been shown to be of value in the treatment of pernicious anaemia in man are: vitamin B_{12} (cyanocobalamin) which is a growth factor for *Lactobacillus leichmannii*; pteroylglutamic acid (probably folic acid) which is a growth factor for *Streptococcus faecalis*; 5-formyl-5: 6: 7: 8-tetrahydropteroylglutamic acid (the citrovorum factor or probably folinic acid) which is a growth factor for *Strep. faecalis* and for *Leuconostoc citrovorum*. The metabolic interrelationships of these factors are uncertain and have already been discussed (Girdwood, 1952*a*, *b*).

The present investigation commenced with a study by microbiological assay methods of the content of vitamin B_{12} and of growth factors for *Strep. faecalis* in the tissues of a patient who died of untreated pernicious anaemia. Later, published work suggested that the citrovorum factor is of importance in relation to haemopoiesis (Nichol & Welch, 1950), and accordingly we commenced studies of the amounts of citrovorum factor in human tissues. Swendseid, Bethell & Ackermann (1951) have introduced a differential microbiological assay procedure for measuring the extent to which the apparent folic acid content of tissues is, in fact, due to citrovorum factor. By a similar approach, and using autopsy material, we have found that in certain human tissues the whole of the material with folic acid activity is citrovorum factor. This is in keeping with the findings of Swendseid *et al.* (1951) who carried out their investigations on fresh mouse livers.

EXPERIMENTAL

Various tissues and organs were obtained at autopsy from the following patients.

Case I. A man aged 65, was admitted to hospital with severe untreated pernicious anaemia diagnosed on the clinical and haematological findings which included sternal puncture. The haemoglobin level was 5·7 g./100 ml., R.B.C. 1·95 millions/cu.mm. Before treatment commenced the patient died suddenly of a coronary thrombosis.

Case II. A woman aged 79, was admitted to hospital with untreated pernicious anaemia (Hb 5·3 g./100 ml., R.B.C. 1·35 millions/cu.mm.). She was given 2 ml. of liver extract on admission (containing about 6 μg. of vitamin B_{12}/ml.) and the following day 100 μg. of vitamin B_{12} together with a transfusion of a pint of blood. She died on the third day in hospital through having aspirated her vomitus into the trachea and bronchi. At the time of death the marrow was not completely converted to the normoblastic state.

Case III. A woman aged 57 was admitted to hospital with hypertensive cardiac failure. There was collapse of the left lung and at autopsy a bronchial carcinoma was found to be present. No visible tumour deposits were present in the tissues that were used for assay. The patient was not anaemic.

Case IV. A man aged 75 was admitted to hospital with a cerebral thrombosis and died the following day. There was no anaemia.

In addition, a sample of liver weighing approximately 2 g. was obtained at operation from a patient (case V) who was undergoing a partial gastrectomy operation for peptic ulcer.

METHODS

All pH estimations were made with glass electrodes.

Buffer solutions

Stock solutions used for the preparation of buffer solutions were (i) 27·228 g. KH_2PO_4 dissolved in distilled water and made up to 1 l. (ii) 35·612 g. $Na_2HPO_4.2H_2O$ dissolved in distilled water and made up to 1 l. 0·2 M-Phosphate buffer solution at pH 7·5 was prepared by mixing 84·2 ml. of solution (i) with 15·8 ml. of solution (ii). 0·2 M-Phosphate buffer solution at pH 6·8 was prepared by mixing equal parts of solutions (i) and (ii).

Preparation of tissues

Tissue extractions were performed by two methods in each instance.

Method A. Approximately 1 g. of tissue was homogenized in 10 ml. of 0·2 M-phosphate buffer solution at pH 7·5. There were added a further 10 ml. of the same buffer solution and the sample was incubated at 37° for 24 hr. The material was then steamed for 10 min. at 100° and filtered. The volume was made up to 50 ml., the final pH being adjusted to 6·8 with 0·1 N-HCl.

Method B. To approximately 1 g. of tissue there were added 20 mg. of pancreatin which itself was found to have negligible growth-promoting activity for the test organisms. Then 10 ml. of 0·2 M-phosphate buffer at pH 6·8 were added and the further steps carried out as in method *A*, the buffer used throughout being of pH 6·8. The final pH was 6·8.

Vitamin assay procedures

Vitamin B_{12}. The content of this was measured using a modification of the method of Hoffmann, Stokstad, Hutchings, Dornbush & Jukes (1949), with *Lb. leichmannii* as test organism. Thioglycollic acid was not included in the medium, the samples instead being added aseptically to the autoclaved medium. It has recently been shown (Pierce, Page, Stokstad & Jukes, 1949) that related forms other than vitamin B_{12} itself occur in nature, and that these forms differ in that they lack a cyano group. For this reason, in the later stages of the investigation, the samples were treated with NaCN to convert alternative forms to cyanocobalamin. This was done after homogenization and incubation.

It is known that certain substances other than vitamin B_{12}, notably various deoxyribosides, will support the growth of *Lb. leichmannii* under the conditions of the assay. Correction for this effect was made by destroying the vitamin B_{12} with alkali and re-assaying for growth activity.

Pteroylglutamic acid and citrovorum factor. The growth of *Strep. faecalis* in a medium deficient in pteroylglutamic acid is stimulated either by pteroylglutamic acid or by citrovorum factor, whereas under the conditions of the assay the growth of *L. citrovorum* depends only upon citrovorum factor and not upon pteroylglutamic acid.

In the earlier stages of the investigation, estimations of combined pteroylglutamic acid and citrovorum factor were made using *Strep. faecalis* and the medium of Teply & Elvehjem (1945). Measurement of growth was by estimation of turbidity. Later, when assays of citrovorum factor were performed these were done by a differential assay method as described by Swendseid *et al.* (1951). Thus, assays of citrovorum factor alone were made with *L. citrovorum* as test organism. For this a modification of the method of Sauberlich & Baumann (1948) was employed, acid-hydrolysed casein being used and thymidine added to the medium since the latter substance reduces the requirement of *L. citrovorum* for citrovorum factor (Broquist, Stokstad & Jukes, 1950). The Ca salt of citrovorum factor, supplied by the kindness of Dr H. Broquist of Lederle Laboratories, Inc., New York, was used to give the standard curves, and concentrations were measured in terms of citrovorum-factor activity.

At the same time assays were performed using *Strep. faecalis* as test organisms, with two series of standard curves produced by pteroylglutamic acid and citrovorum factor, respectively. It was thus possible to measure 'total apparent pteroylglutamic acid activity' (FAA) and, at the same time 'apparent citrovorum activity' (ACF) with *Strep. faecalis*. Since citrovorum factor has been used as a standard in both the *L. citrovorum* and *Strep. faecalis* assays, if the activity of a given specimen in stimulating *Strep. faecalis* was greater than its activity in stimulating *L. citrovorum*, pteroylglutamic acid must have been present. If the results were the same in each instance, only citrovorum factor was present. Since the true content of citrovorum factor is known from the *L. citrovorum* assay, and there are both pteroylglutamic acid and citrovorum factor standards in the *Strep. faecalis* assay, it is possible to calculate to what extent citrovorum factor is falsely showing itself as total apparent pteroylglutamic acid activity and hence to calculate true pteroylglutamic acid content.

These assays cannot differentiate between free and conjugated forms of the various haematopoietic factors.

Under the conditions of the assay reported here, the stimulation of growth of *Strep. faecalis* produced by 1·0 mg. of citrovorum factor (calculated as the free acid) was equivalent to that produced by 0·58–0·78 mg. of pteroylglutamic acid. There were variations with individual assays but the results were consistently within this range. This is in keeping with the findings of Broquist (1951), but differs from the experience of Swendseid *et al.* (1951) who used folinic acid provided by Dr William Shive as standard, and found that 1·0 mg. of folinic acid had the growth-promoting activity of 1·5 mg. of pteroylglutamic acid.

RESULTS

Significance of assay procedures

Nichol & Welch (1950) have shown that pteroylglutamic acid may be converted to citrovorum factor in tissues under certain conditions, and hence we have followed the practice of Swendseid *et al.* (1951) of adding pteroylglutamic acid to samples of

Table 1. *Recovery of pteroylglutamic acid and citrovorum factor added to the buffer in which human tissues were homogenized*

Organ	Method of extraction	Supplement added to homogenate	Citrovorum factor activity (*L. citrovorum* assay) (μg./g.)	Pteroylglutamic acid activity (*Strep. faecalis* assay) (μg./g.)
Liver	*B*	None	0·67	0·61
	B	10 μg. PGA/g.	0·68	9·8
	B	4·38 μg. CF/g.	4·3	4·1
	A	None	0·45	0·47
	A	10 μg. PGA/g.	0·44	9·0
	A	4·38 μg. CF/g.	4·1	3·9
Liver	*B*	None	1·0	0·79
	B	0·922 μg. CF/g.	1·8	1·4
Kidney	*B*	None	0·22	0·10
	B	0·8 μg. PGA/g.	0·25	0·89
	B	0·922 μg. CF/g.	0·97	0·73

Table 2. *Results of repeated assays on different specimens of same organs*

Case	Organ	Method of estimation	Vitamin B_{12} content (*Lb. leichmannii* assay) (μg./100 g.)	Growth factors for *Strep. faecalis* (as PGA) (μg./100 g.)	Citrovorum factor content (*L. citrovorum* assay) (μg./100 g.)
III	Liver	*B*	22, 29, 25	160, 170, 160	150, 160, 180
		A	23, 26, 24	170, 150	150, 150
IV	Liver	*B*	58, 53, 50	390, 410	530, 520

certain of the tissues prior to incubation to see whether such conversion occurred under the conditions of our assay. When pteroylglutamic acid had been added to the fluid in which the tissues were homogenized, there was no increase in the ability of samples to stimulate the growth of *L. citrovorum* although the added pteroylglutamic acid could be recovered to the extent of 80–98 %, as shown by the *Strep. faecalis* assay (Table 1). When citrovorum factor was added before homogenization, recoveries were equally satisfactory, and hence there was no evidence of conversion of pteroylglutamic acid to citrovorum factor or citrovorum factor to pteroylglutamic acid.

In all instances, assays were performed at least in duplicate, usually in quadruplicate, and the accuracy of the method was checked by performing repeated assays on several specimens of the same tissue, separately weighed and homogenized (Table 2).

Content of vitamin B_{12} *and of growth factors for* Streptococcus faecalis *in a patient with untreated pernicious anaemia*

The results of this investigation are given in Table 3. It will be seen that no vitamin B_{12} was detected in any organs or tissues. In the assay method employed each tube contained 10 ml. of fluid of which 5 ml. comprised double strength medium. When 0·2 μmg. of vitamin B_{12} was added to a series of such assay tubes containing 0·5, 1, 2, 3 and 4 ml. respectively of extracts of each of the tissues of this patient, prepared as in method *B*, but brought to a final dilution of 100 ml., growth occurred equally in all tubes except in the case of the extract of skeletal muscle where it was progressively less with increasing content of tissue extract. Since

Table 3. *Vitamin* B_{12} *and total apparent pteroylglutamic acid content of the tissues and organs of a patient with untreated pernicious anaemia case I*

(The vitamin B_{12} content (*Lb. leichmannii* assay) without cyanide was nil in all cases.)

Organ or tissue	Method of extraction	Total apparent folic acid activity (*Strep. faecalis* assay) (μg./100 g.)
Liver	*B*	83
	A	90
Kidney	*B*	24
	A	5·5
Spleen	*B*	14
	A	8·9
Lung	*B*	Negligible
	A	Negligible
Brain	*B*	5·4
	A	—
Stomach	*B*	Negligible
	A	Negligible
Intestine	*B*	14
	A	3·9
Tongue	*B*	12
	A	3·4
Muscle	*B*	8·2
	A	4·1

the amount of growth in all other instances was equal to that produced by 0·2 μmg. of vitamin B_{12} in water, it appears that in most of the tissues no inhibitors were present to prevent the growth-stimulating effect of vitamin B_{12} for *Lb. leichmannii*.

what was found in cases III and IV, an appreciable part of the apparent pteroylglutamic acid content was not due to citrovorum factor. The patient received approximately 112 μg. of vitamin B_{12} by injection before death. The liver weighed 1120 g.

Table 4. *Content of haemopoietic factors in the tissues of non-anaemic human patients*

Case no.	Organ or tissue	Method of extraction	Vitamin B_{12} content (*Lb. leichmannii* assay) Without cyanide (μg./100 g.)	Vitamin B_{12} content (*Lb. leichmannii* assay) After cyanide treatment (μg./100 g.)	Total apparent folic acid activity (FAA) *Strep. faecalis* assay (μg./100 g.)	Apparent citrovorum-factor activity (ACF) *Strep. faecalis* assay (μg./100 g.)	True citrovorum-factor activity (CF) *L. citrovorum* assay (μg./100 g.)	True folic acid activity (PGA) (μg./100 g.)
III	Liver	*B*	25	28	130	170	160	Negligible
		A	24	28	138	160	150	Negligible
	Kidney	*B*	16	15	79	84	90	Nil
		A	7·1	7·1	16	16	14	Negligible
	Spleen	*B*	3·9	4·7	39	47	47	Nil
		A	4·6	5·0	6·0	7·8	9·9	Nil
	Lung	*B*	5·1	5·2	65	76	42	30
		A	4·0	4·2	33	36	9·4	26
	Brain	*B*	9·1	10	38	48	14	27
		A	7·0	8·7	12	18	1·7	11
	Muscle	*B*	8·3	8·4	39	42	24	21
		A	7·8	6·4	7·5	13	3·9	4·5
	Skin	*B*	1·3	1·5	24	25	7·8	18
		A	1·4	1·2	15	17	Negligible	15
IV	Liver	*B*	54	67	400	520	520	Nil
		A	55	74	400	510	540	Nil
	Kidney	*B*	25	32	38	38	40	Nil
		A	33	37	34	41	42	Nil
	Spleen	*B*	36	39	71	78	29	49
		A	12	21	16	19	5·8	11
	Brain	*B*	22	22	17	20	3·7	14
		A	5·9	6·1	Negligible	Negligible	Negligible	Negligible
	Stomach	*B*	22	30	38	40	23	21
		A	6·2	7·8	21	20	8·1	15
	Intestine	*B*	28	27	23	26	3·1	20
		A	15	16	20	20	5·3	16
	Muscle	*B*	11	16	18	20	17	5·4
		A	9·9	12	16	13	9·1	9·7
V	Liver	*B*	50	—	430	550	500	41
		A	46	—	310	390	300	81

Content of vitamin B_{12}, pteroylglutamic acid and citrovorum factor in the organs and tissues of control patients

The results of this investigation are given in Table 4. It will be seen that in the liver and kidney almost all the apparent pteroylglutamic acid was, in fact, citrovorum factor, but that in most instances the other organs and tissues contained both of these forms.

Content of haemopoietic factors in the organs and tissues of a pernicious anaemia patient at the commencement of treatment

The results of this investigation are given in Table 5. It will be seen that here, in contrast to

and the kidneys each weighed 120 g. If the highest figure obtained for B_{12} content (method *B*, treated with sodium cyanide) is taken to be the correct one, then the liver contained 260 μg. of vitamin B_{12} and in the kidneys there was a further 17·8 μg.

DISCUSSION

These results give an indication of the occurrence of vitamin B_{12}, pteroylglutamic acid and citrovorum factor in human tissues and indicate that in the liver and kidneys the apparent pteroylglutamic acid is largely citrovorum factor. It is possible, but unlikely, that this was due to post-mortem conversion of pteroylglutamic acid to citrovorum factor because (1) when pteroylglutamic acid was

incubated with liver or kidney, which did not appear themselves to contain appreciable amounts of this substance, conversion to citrovorum factor did not occur; (2) similar results were found by Swendseid *et al.* using fresh mouse livers; (3) in the liver obtained at surgical operation, growth of *Strep. faecalis* was very largely due to citrovorum factor; and (4) in the liver of the pernicious anaemia patient (case II) there was no increase of the percentage of growth activity for *Strep. faecalis*, due to citrovorum factor, after the organ had been kept frozen for 4 months. Unfortunately, it was not possible to carry out assays for citrovorum factor at the time when the tissues of case I were investigated, since at that time citrovorum factor for use as a standard was not available.

though Cuthbertson (1951) states that cyanide is more effective if added prior to homogenization.

No vitamin B_{12} was found in the tissues and organs of case I, and except with the extract of muscle there was no evidence for the presence of any inhibitors that would prevent added vitamin B_{12} from supporting the growth of the test organism. The findings for the vitamin B_{12} content of the organs in case II are more difficult to interpret. It is known that when vitamin B_{12} is given by injection in doses of 100 μg. or more, there is a considerable and rapid excretion of the vitamin in the urine, and yet the combined vitamin B_{12} contents of the liver and kidneys appear to be greater than the amount that was injected. There is already some evidence that the tissues and organs of patients with pernicious

Table 5. *Content of haemopoietic factors in the organs and tissues of a pernicious anaemia patient at commencement of treatment. Case II*

Organ	Method of extraction	Vitamin B_{12} content (*Lb. leichmannii* assay) Without cyanide (μg./100 g.)	Vitamin B_{12} content (*Lb. leichmannii* assay) After cyanide treatment (μg./100 g.)	Total apparent folic acid activity (FAA) *Strep. faecalis* assay (μg./100 g.)	Apparent citrovorum-factor activity (ACF) *Strep. faecalis* assay (μg./100 g.)	True citrovorum-factor activity (CF) *L. citrovorum* assay (μg./100 g.)	True folic acid activity (PGA) (μg./100 g.)
Liver	*B*	14	23	120	140	79	62
	A	3·2	4·1	65	66	26	45
Kidney	*B*	5·0	7·3	46	43	46	Nil
	A	2·6	2·9	11	15	12	2·0
Skin	*B*	0·27	—	11	16	1·5	9·7
	A	0·26	Negligible	Negligible	Negligible	Negligible	Negligible

It appears that the liver of case II, a pernicious anaemia patient at the commencement of treatment, differs from that of cases III and IV in that pteroylglutamic acid itself was present to an appreciable extent either in a free or conjugate form. However, further work on the livers of untreated patients with megaloblastic anaemia and on many more normal livers is necessary before any claim can be made that one of the metabolic errors in pernicious anaemia is a failure in the conversion of pteroylglutamic acid to citrovorum factor. The fact that the therapeutic dosage of pteroylglutamic acid and of citrovorum factor is similar (Davidson & Girdwood, 1951) suggests that this cannot be the primary metabolic fault.

It will be noted that in common with other workers we found that with certain tissues the content of haemopoietic factors was greater when pancreatin extraction was used, and there is no reason to believe that the higher figure in each instance is not the correct one. In most instances the addition of sodium cyanide after homogenization caused little increase in the content of vitamin B_{12} suggesting that little vitamin B_{12b} was present, anaemia in relapse are not always completely devoid of vitamin B_{12} (Girdwood, 1951; Drouett, Wolff & Rauber, 1951), but if this is the true explanation it would appear reasonable to assume that the vitamin B_{12} present in the tissues of such patients is in a 'bound' form that is not available for haemopoiesis. An alternative interpretation would be that some other substance is present that stimulates the growth of *Lb. leichmannii* under the conditions of the assay despite the use of a technique that includes alkaline hydrolysis.

SUMMARY

1. An account is given of the distribution of vitamin B_{12}, pteroylglutamic acid and citrovorum factor in the tissues and organs at autopsy of two non-anaemic patients. The liver and kidneys contained relatively large amounts of citrovorum factor, but no pteroylglutamic acid.

2. The tissues and organs of a patient with untreated pernicious anaemia contained no vitamin B_{12}. In another pernicious anaemia patient who died at the commencement of treatment, the amount

of vitamin B_{12} recovered appeared to be greater than the amount given. The liver of this patient contained appreciable amounts of both pteroylglutamic acid and citrovorum factor.

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REFERENCES

Broquist, H. P. (1951). Personal communication.
Broquist, H. P., Stokstad, E. L. R. & Jukes, T. H. (1950). *J. biol. Chem.* **185**, 399.
Cuthbertson, W. F. J. (1951). Personal communication.
Davidson, L. S. P. & Girdwood, R. H. (1951). *Lancet*, **2**, 1193.
Drouett, P. L., Wolff, K. & Rauber, G. (1951). Quoted in *J. Amer. med. Ass.* **146**, 493.
Girdwood, R. H. (1951). *Brit. J. Nutrit.* **5**, xi.
Girdwood, R. H. (1952*a*). *Blood*, **7**, 77.
Girdwood, R. H. (1952*b*). *Brit. J. Nutrit.* (in the Press).
Hoffmann, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbush, A. C. & Jukes, T. H. (1949). *J. biol. Chem.* **181**, 635.
Nichol, C. A. & Welch, A. D. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 403.
Pierce, J. V., Page, A. C. jun., Stokstad, E. L. R. & Jukes, T. H. (1949). *J. Amer. chem. Soc.* **71**, 2952.
Sauberlich, H. E. & Baumann, C. A. (1948). *J. biol. Chem.* **176**, 165.
Swendseid, M. E., Bethell, F. H. & Ackermann, W. W. (1951). *J. biol. Chem.* **190**, 791.
Teply, L. J. & Elvehjem, C. A. (1945). *J. biol. Chem.* **157**, 303.

Some Observations on the Hydrolysis and Extraction of Formaldehydogenic Corticosteroids

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Of the methods recently reviewed by Dorfman (1950) and Callow (1950) for estimating urinary corticosteroids only a few have been generally adopted. In the bioassay procedure of Venning, Kazmin & Bell (1946) use is made of the ability of certain 11-oxysteroids to promote the deposition of glycogen in the liver of adrenalectomized mice. The chemical procedures are of two main types. The first depends on the reducing properties of steroids with a primary or secondary α-ketol group, or an αβ-unsaturated 3-keto group or both. Talbot, Saltzman, Wixom & Wolfe (1945) used this property to reduce cupric ions, and Heard & Sobel (1946) phosphomolybdic acid. Since urine may contain a wide variety of reducing agents, the specificity of the assay depends upon the efficiency of the extraction and the purification of the extract. The second chemical method depends upon the liberation of formaldehyde when steroids with a free terminal vicinal glycol or hydroxyketone group are oxidized with periodic acid (Lowenstein, Corcoran & Page, 1946; Daughaday, Jaffe & Williams, 1948; Corcoran, Page & Dustan, 1950). The liberated formaldehyde reacts with chromotropic acid to give a lavender colour.

More recently, Zaffaroni, Burton & Keutman (1950) and Burton, Zaffaroni & Keutman (1951) have evolved a paper partition chromatographic procedure which permits separation of urinary corticoids into individual components. Such fractionation is an advance on the less specific methods, but unfortunately permits only semi-quantitative determinations.

All these methods require the extraction of corticosteroids from urine. Hormones which are excreted in the free form as neutral lipid-soluble substances are readily extracted by organic solvents. Others, however, are excreted in water-soluble conjugated form as sulphates and glucuronides (Klyne, 1946; Klyne & Marrian, 1945; Lieberman & Dobriner, 1948) and these must be hydrolysed before extraction. In the method of Talbot *et al.* (1945) the urine is extracted without adjusting the pH, and the steroids obtained are

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probably only those excreted in the free form. Heard & Sobel (1946) have found that the pH at which urine is extracted markedly influences the yields of both glycogen-depositing and total reducing substances. On acidification to pH 1 and immediate extraction the biological titre by Venning's method was approximately twice that obtained on extracting a sample of the same urine at pH 7. Likewise the reducing power was greater with increasing hydrogen-ion concentration during extraction, the value at pH 2 being two to three times that at pH 7. Lieberman & Dobriner (1948) found that the yield of compounds possessing glycogen-depositing activity could be increased by hydrolysis at pH 1 at room temperature for 24 hr. By model experiments they showed that when sulphates of certain 17-ketosteroids were continuously extracted at room temperature for 24–48 hr. at pH 1, the conjugate was completely hydrolysed and the free steroid recovered quantitatively.

That the optimum conditions for hydrolysis have not been fully considered is shown by the fact that Talbot *et al.* (1945) use extracts of unacidified urine; Lowenstein *et al.* (1946), Heard & Sobel (1946) and Daughaday *et al.* (1948) extract the urine immediately after acidification, and Mason & Sprague (1948) allow the urine to stand at pH 1 for 1–3 days before extraction. None of these procedures, with the possible exception of the last, would be likely to hydrolyse sulphate or glucuronide conjugates.

The hydrolysis of glucuronide conjugates has been achieved with β-glucuronidase prepared from either animal viscera (Fishman & Talalay, 1947) or *Escherichia coli* (Kinsella, Doisy & Glick, 1950). By this procedure the yield of formaldehydogenic steroids has been 5- to 20-fold greater than following acidification of urine (Corcoran *et al.* 1950; Corcoran, Dustan & Page, 1951; Kinsella *et al.* 1950; Cox & Marrian, 1951).

Marrian and his associates have recently reported their investigations on the conjugated adrenocortical steroids in urine (Paterson, Cox & Marrian, 1950; Paterson & Marrian, 1951; Cox & Marrian, 1951). They have found two types. The first conjugate was readily extractable with chloroform from acidified but not from neutral urine, and was rapidly hydrolysed at pH 1 to yield acid-labile steroid. The unhydrolysed conjugated compound could be recovered from the chloroform phase by extracting with aqueous sodium bicarbonate and could then be hydrolysed to the free compound in 1–3 hr. at room temperature and pH 3–4. The free steroid was stable at this pH for at least 6 hr. but was slowly destroyed at pH 1. This conjugate was not a glucuronide because it could not be hydrolysed with β-glucuronidase. The second conjugate was not extracted by chloroform even from acidified urine but was slowly hydrolysed at pH 1. It was obtained by extracting with *n*-butanol acidified urine which had previously been extracted with chloroform to remove free steroids and chloroform-soluble conjugates. The butanol phase was found to contain a glucuronide conjugate of corticosteroids which could be removed with aqueous sodium hydroxide and hydrolysed with β-glucuronidase to yield formaldehydogenic steroid in amounts about ten times greater than could be obtained by hydrolysis for 24 hr. at pH 1 and 37°.

In view of the present uncertainty as to the best method of hydrolysing urinary corticosteroids, studies were undertaken to define in what different conjugated forms the steroids were excreted, and to devise procedures which would ensure their hydrolysis and permit extraction of the free compounds liberated. Unless such methods are used, it may be argued that the quantity of steroid obtained by extracting acidified or unacidified urine only represents a proportion of the total excretion, and may not be a true reflexion of adrenocortical activity.

METHODS

Material. 24 hr. Urine specimens were collected without preservative from healthy males aged 25–35. In experiment (*j*) urine was obtained from three males suffering from rheumatic fever.

Apparatus. Glassware was cleaned by soaking in a hot solution of detergent ('Calgonite' or 'Haemosol'), washed in tap water and rinsed in distilled water. It was dried by draining at room temperature. The same apparatus was used throughout, and the dimensions of the vessels were the same at each stage of the procedure.

Hydrolysis. Urine for acid hydrolysis was adjusted to pH 1 (thymol blue) with conc. HCl; that for extraction at neutral pH was adjusted to pH 7·4 (neutral red) with N-HCl or N-NaOH; that for glucuronidase hydrolysis was adjusted to pH 4·5 (bromocresol green) with N-HCl.

β-Glucuronidase was prepared from rabbit livers and spleens after the method of Fishman & Talalay (1947). The enzyme obtained after the second precipitation with $(NH_4)_2SO_4$ was dissolved in the smallest possible volume of water and had an activity of 19000 Fishman units/ml. (1 unit liberates 1 μg. phenolphthalein in 1 hr. at 38° from phenolphthalein mono-β-glucuronide at pH 4·5 in 0·1 M-acetate buffer, Talalay, Fishman & Huggins, 1946). The batch of enzyme concentrate was stored at below −15°, and by repeated assay was found not to have lost any of its activity in 9 months. Urine for glucuronidase hydrolysis was adjusted to pH 4·5 and 5 ml. of 1·0 M-acetate buffer added per 100 ml. urine. 5000 units of enzyme were added to each 100 ml. urine. It was found that doubling or trebling the amount of glucuronidase did not increase the yield. The effectiveness of smaller amounts of enzyme was not investigated. The urine was incubated at 37–38° for 48 hr.

Extraction. Throughout, 200 ml. samples of urine were assayed. The samples were extracted by hand three times with 50 ml. portions of redistilled A.R. $CHCl_3$ or ether. The solvent phase was washed twice with 50 ml. portions of 5% (w/v) Na_2CO_3 and with 50 ml. portions of water until neutral. The carbonate and water washings were each back

extracted with 25 ml. solvent. The solvent phase was dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure at 40°.

Choice of solvent. Talbot *et al.* (1945) recommended the use of $CHCl_3$ for extracting neutral lipid-soluble steroids from urine. They found that $CHCl_3$ effected almost quantitative recovery, but ether proved less efficient and only 51% of 11-dehydro-17-hydroxycorticosterone and 65% of 17-hydroxycorticosterone were recovered when 0·2 mg. of either of these steroids was dissolved in 50 ml. water and shaken for 3 min. with an equal volume of solvent. Heard & Sobel (1946), by trial extractions of 11-deoxycorticosterone from water and urine, found that three extractions with ether alone recovered 80%, whereas an ether-$CHCl_3$ mixture (4:1) gave almost quantitative recovery. There seems little doubt that these steroids are more soluble in $CHCl_3$ than in ether, but the difference is not so great as to preclude the use of ether if it has other advantages. Emulsion formation is often excessive when extracting glucuronidase-hydrolysed urine with chloroform owing to the proteinous nature of the enzyme. This difficulty is considerably reduced with ether, which was mainly used in these experiments. As shown in Table 1, the yield of formaldehydogenic substances from two samples of the same urine does not differ significantly when the extraction is made with chloroform or with ether.

Table 1. *Comparison between the yield of FS when samples of urine are extracted with ether and with chloroform*

Urine no.	Hydrolysis	*FS* (mg. compound E/24 hr.) Extracted with ether	Extracted with $CHCl_3$
I	pH 1 and immediate extraction	1·6	1·5
II	Extracted at pH 7·4	0·7	0·8
III	pH 1 and immediate extraction	1·9	1·9
IV	β-Glucuronidase	4·5	4·9
V	β-Glucuronidase	19·4	18·5
VI	β-Glucuronidase	12·0	8·6*

* Bad emulsion formation.

Chemical method. The urinary extracts were dissolved in 10 ml. of 10% (v/v) ethanol, and 6 ml. transferred to a 30 ml. distilling flask. (When amounts of formaldehydogenic steroid greater than 4 mg./24 hr. were encountered, the extracts were diluted with larger volumes of ethanol. Smaller volumes were used when less than 1 mg./24 hr. was expected.) A reagent blank of 6 ml. 10% ethanol and standards of 11-dehydro-17-hydroxycorticosterone (compound E) in 6 ml. 10% ethanol were run concurrently in distilling flasks of the same dimensions. Oxidation with periodic acid, removal of excess oxidant with $SnCl_2$, the distillation of formaldehyde and the colour reaction with chromotropic acid were carried out as described by Daughaday *et al.* (1948). With the reagent blank set at 100% transmission, the unknowns and standards were read in a Coleman 'Universal' spectrophotometer at 550 mμ. The formaldehyde liberated from 100 μg. compound E gave an optical density of 0·241 with minor variations from day to day. All results are expressed as mg. of compound E excreted in 24 hr.

Controls obtained by extracting 200 ml. water with $CHCl_3$ and with ether gave a colour equivalent to 11 and 9 μg. respectively of compound E. These would have affected the values for a 24 hr. urine specimen of 1400 ml. by 0·08 and 0·06 mg. When 200 ml. water, buffered with 10 ml. 1·0 M-acetate buffer and containing 10 000 units of glucuronidase concentrate, was incubated for 48 hr. and then extracted with $CHCl_3$ or ether, values of 27 and 20 μg. compound E were obtained. These would have affected the values for a 24 hr. urine specimen by 0·19 and 0·14 mg. No corrections have been made for these controls.

RESULTS

(*a*) *Formaldehydogenic substances extracted at* pH 7·4 *compared with those extracted immediately after acidification to* pH 1

One sample of each urine specimen was extracted at pH 7·4, and another sample after acidification to pH 1. In most instances acidification increased the yield of formaldehydogenic substances (*FS*) (Table 2).

Table 2. *Comparison between the yield of FS obtained after extraction at* pH 7·4 *and after extraction immediately following acidification to* pH 1

Urine no.	*FS* (mg. compound E/24 hr.) Extracted at pH 7·4	Extracted immediately after acidification to pH 1
I	0·5	1·5
II	0·8	1·5
III	1·3	1·9
IV	2·7	2·8
V	1·2	5·4
VI	0·3	0·8

Comment. Similar observations have been made by Heard & Sobel (1946) and by Paterson *et al.* (1950). Acidification may increase the yield by (*a*) causing more complete extraction of the free steroids, (*b*) permitting extraction of a conjugated steroid, or (*c*) by hydrolysing a steroid conjugate and then allowing extraction of the free compound liberated. The observations of Paterson & Marrian (1951) suggest that some, if not all, of the increase is due to the rapid hydrolysis at pH 1 of a conjugated compound, which, however, in the conjugated form is also extracted by chloroform from acidified urine (see Exp. *c*).

(*b*) *FS extracted first at* pH 7·4 *and then at* pH 1 *compared with FS extracted at* pH 1 *only*

The quantity of *FS* obtained from a urinary sample first extracted at pH 7·4 and then extracted again immediately after acidification was compared with the yield when another sample was extracted only after acidification (Table 3). A greater yield

was not obtained by the double extraction procedure. In the first two specimens (urines III and IV) the increment of *FS* obtained after acidification was not large relative to the amount extracted at neutral pH, whereas in the third specimen (urine VI) the amount of *FS* determined. Another sample of the same urine was extracted immediately after acidification to pH 1. The results (Table 4) indicate that acidification increased the yield of *FS* 0·4 mg. above the amount obtained from urine extracted at pH 7·4. Despite this increase the alkali washings contained 0·3 mg. when hydrolysed without β-glucuronidase and 0·4 mg. when hydrolysed with the enzyme.

Table 3. *FS obtained from urinary samples first extracted at* pH 7·4 *and then re-extracted at* pH 1 *compared with FS obtained from samples extracted only at* pH 1

	FS (mg. compound E/24 hr.)		
	Urine III	Urine IV	Urine VI
Extracted at pH 7·4	1·3	2·7	0·3
Re-extracted immediately after acidification to pH 1	0·4	0·3	0·4
Total	1·7	3·0	0·7
Extracted only at pH 1	1·9	2·8	0·8

0·3 mg. was obtained at pH 7·4 and 0·4 mg. after acid hydrolysis. This experiment also shows that no destruction of the free steroids present in neutral urine is detectable when the urine is acidified to pH 1 and extracted immediately.

(*c*) *The role of chloroform-soluble conjugates in increasing the yield of FS from acidified urine*

In Exp. (*a*) it was found that acidification of urine followed by immediate extraction increased the yield of *FS*. It was suggested that this increase may be due to the rapid hydrolysis of a conjugated compound such as the chloroform-soluble conjugate described by Paterson & Marrian (1951). However Heard & Sobel (1946) and Paterson *et al.* (1950) have shown that in some instances no increase is obtained if the acidified urine is neutralized before it is extracted. The latter authors suggested that the explanation for this was that the chloroform-soluble conjugate could not be extracted from neutral urine. It was only extractable from acidified urine and the conjugate could be recovered from the chloroform phase by extracting with aqueous sodium bicarbonate. It could then be hydrolysed to the free compound in 1–3 hr. at room temperature and pH 3–4.

The following experiment was done to determine how much of the chloroform-soluble conjugate, after it had been subjected to acid hydrolysis, could be recovered in the conjugated form from the chloroform phase. A sample of urine was extracted with chloroform at pH 7·4 and then acidified to pH 1 and extracted with chloroform again. The alkali washings from the second extraction procedure were freed of organic solvent by bubbling nitrogen through, adjusted to pH 4·5 and buffered with 1·0M-acetate buffer. One half of the washings was incubated with β-glucuronidase; the other half was incubated without enzyme. After 24 hr. the alkali washings were extracted with chloroform and

Table 4. *Effect of acid on the hydrolysis of chloroform-soluble conjugates, and their subsequent recovery from alkali washings*

	FS (mg. compound E/24 hr.)
First sample:	
(*a*) Extracted at pH 7·4	1·3
(*b*) Extracted again immediately after acidification to pH 1	0·4
Total	1·7
Extraction of the alkali washings obtained during procedure (*b*)	
Hydrolysed with β-glucuronidase	0·4
Hydrolysed without enzyme	0·3
Second sample:	
Extracted immediately after acidification to pH 1	1·9

Comment. If the increased yield after acidification is due to the hydrolysis of a chloroform-soluble conjugate or conjugates, this hydrolysis only affected a proportion of the conjugate, the remainder passing into the alkali washings. The amount of *FS* in the alkali washings was approximately the same whether the washings were hydrolysed with or without β-glucuronidase. It is not possible to say, therefore, whether the conjugate was a glucuronide or not.

(*d*) *The solubility of the chloroform-soluble conjugate in ether*

Two samples of urine were acidified to pH 1. One was extracted immediately with ether, the other with chloroform. The alkali washings of the ether and chloroform extracts were separately adjusted to pH 4·5, buffered with 1·0M-acetate buffer and incubated for 24 hr. at 37°. The alkali washings were then extracted with chloroform. The ether and chloroform extracts each contained 1·9 mg. of *FS*. The alkali washings of the ether extract contained 0·2 mg. and those of the chloroform extract 0·3 mg. (Table 5). This suggests that the chloroform-soluble conjugate is also soluble in ether.

(*e*) *The effect of acid on FS*

To determine whether *FS* are acid-labile a sample of urine was extracted with ether immediately after acidification to pH 1. The ether phase

was washed with sodium carbonate and water and then divided into two halves. One half was taken to dryness under reduced pressure and the residue dissolved in 20 ml. 10% (v/v) ethanol. The ethanolic solution was adjusted to pH 1 with hydrochloric acid and allowed to stand at room temperature for 24 hr. before being extracted with chloroform. The other half was treated similarly except that the ethanolic solution was not acidified. The amount of *FS* in the acid-treated sample was 0·4 mg. and in the unacidified sample 1·3 mg. A repetition of this experiment with another urine specimen gave 0·2 mg. for the acidified solution and 0·6 mg. for the unacidified solution. These observations indicate that certain *FS* extractable from urine are acid-labile.

Table 5. *Comparison between the solubility of the chloroform-soluble conjugate in chloroform and in ether*

	FS (mg. compound E/24 hr.)	
	Ether extract	$CHCl_3$ extract
Urine extracted immediately after acidification to pH 1	1·9	1·9
Alkali washings incubated at pH 4·5 and then extracted with $CHCl_3$	0·2	0·3

(f) The effect of prolonged standing in acid on the yield of FS

The destructive action of acid on *FS* was studied by acidifying two specimens of urine to pH 1 and then extracting with chloroform samples of each immediately, and 4, 12 and 24 hr. after acidification (Table 6). The decreasing yield which occurs as the period of standing in acid is lengthened confirms that prolonged standing at pH 1 destroys certain *FS*.

Table 6. *Effect of prolonging the acid treatment on the yield of FS*

Time after acidification (hr.)	*FS* (mg. compound E/24 hr.)	
	Urine II	Urine III
Immediate	1·5	1·9
4	1·4	1·7
12	0·8	1·0
24	0·4	0·3

Comment. Genest (1951) has also found that prolonged standing in acid caused a decreased yield. On the other hand, Paterson *et al.* (1950) observed that although the yield of *FS* was reduced when urine was extracted 4 hr. after acidification, it rose again when the extraction was performed 12 or 24 hr. later. This increase was attributed to the slow hydrolysis of a conjugated compound.

The following experiments provide further data on the effect of prolonged acidification which, in this work, had reduced the yield of *FS* and in the hands of Cox & Marrian (1951), had increased it. Samples of urine were acidified to pH 1. One was allowed to stand at room temperature for 24 hr. before extraction; the other was extracted immediately after acidification and again 24 hr. later (Table 7). When the urine was extracted immediately after acidification, a further increment was obtained when the extraction was repeated 24 hr. later. This suggests that prolonged acidification causes slow hydrolysis of a conjugated compound. On the other hand, the yield of *FS* was less when the urine was extracted after 24 hr. hydrolysis at pH 1 than when it was extracted immediately after acid fication and again 24 hr. later.

Table 7. *Action of acid in destroying free compounds and slowly hydrolysing a conjugated compound*

	FS (mg. compound E/24 hr.)			
	Urine I	Urine III	Urine VII	Urine IX
First sample:				
Extracted immediately after acidification	1·5	1·9	4·6	2·7
Re-extracted after 24 hr.	0·5	0·3	0·6	1·3
Total	2·0	2·3	5·2	4·0
Second sample:				
Extracted 24 hr. after acidification	0·4	0·3	2·7	2·3

Comment. The yield of *FS* after acidification and extraction 24 hr. later represents the balance between those free compounds remaining after partial destruction and those which have been liberated from a conjugated form. The proportion destroyed varies from one urine to another. In urine I, for example, immediate extraction after acidification gave 1·5 mg. Repeated extraction 24 hr. later gave 0·5 mg. which does not differ from the value (0·4 mg.) obtained when another sample was allowed to stand for 24 hr. at pH 1 before being extracted. In this instance it would seem that most of the free substances and those liberated from the chloroform-soluble conjugate, which amounted to 1·5 mg., were acid-labile and destroyed by prolonged acidification. The same holds for urine III. On the other hand, in urines VII and IX, there was less destruction of the free substances because the values obtained when the urine was extracted after standing at pH 1 for 24 hr. (2·7 and 2·3 mg. respectively) were greater than those when other samples were extracted for the second time (0·6 and 1·3 mg. respectively). It will also be seen that the proportion of free compounds, excreted as such or hydrolysed from the chloroform-soluble conjugate, to that conjugated compound, which is slowly hydrolysed at pH 1, varies from one urine to another.

(g) Further observations on the action of acid in hydrolysing conjugates

A sample of urine was acidified and extracted immediately, yielding 1·5 mg. *FS*. The same sample was allowed to stand at room temperature and pH 1 for 24 hr. when it yielded another 0·5 mg. After a further 24 hr. it was extracted again and then gave 0·3 mg. Finally the same sample was hydrolysed with β-glucuronidase and 4·1 mg. obtained.

In another experiment a sample of urine was extracted at pH 7·4 (2·0 mg.) and again immediately after acidification (0·4 mg.). 4 hr. later, after standing at room temperature and pH 1, it was extracted again (0·5 mg.). 24 hr. after acidification, extraction of the same sample yielded 1·2 mg. Finally, the sample was hydrolysed with β-glucuronidase and yielded 18·5 mg.

Comment. These experiments provide further evidence that prolonged treatment with acid causes slow hydrolysis of a conjugated compound—in the first instance yielding 0·8 mg. and in the second 1·7 mg. Even then the urines contained 4·1 and 18·5 mg. respectively of *FS* conjugated with glucuronide.

(h) Action of β-glucuronidase on the yield of FS

To confirm that hydrolysis with β-glucuronidase increases the yield of *FS*, samples of urine were adjusted to pH 4·5 and buffered. The enzyme preparation was added to one sample; another sample acted as a control. After incubation at 38° for 48 hr. both were extracted without altering the hydrogen-ion concentration. The results (Table 8) indicate that β-glucuronidase hydrolysis causes a considerable increase in the yield of *FS*.

Table 8. *Effect of β-glucuronidase hydrolysis on the yield of FS*

	FS (mg. compound E/24 hr.)	
Urine no.	β-Glucuronidase hydrolysis at pH 4·5	Control hydrolysis at pH 4·5
X	4·1	1·4
XI	12·6	1·5
XII	9·3	1·8

(i) The effect of acid on FS liberated from glucuronide conjugation

The following experiments were designed to determine whether the free substances liberated from glucuronide conjugation were acid-labile. Two samples of urine were hydrolysed with β-glucuronidase. After incubation one was extracted at pH 4·5; the other was acidified to pH 1 before extraction (Table 9). In two instances acidification caused a slight reduction in the yield of *FS* and in a third instance the reduction was more marked. Thus acidification appears to cause some destruction of the liberated compound. No observations were made to determine whether prolonged acidification caused a greater degree of destruction.

Table 9. *Effect of acid on free compounds liberated from glucuronide conjugation*

	FS (mg. compound E/24 hr.) Urine hydrolysed with β-glucuronidase	
Urine no.	Extracted at pH 4·5	Extracted at pH 1
X	4·1	1·7
XI	12·6	10·4
XII	9·3	8·1

(j) Comparison between the yields of FS after acid and β-glucuronidase hydrolysis

One sample of urine was hydrolysed at pH 1 for 24 hr. Another was hydrolysed with β-glucuronidase, and after incubation the urine was made pH 1 immediately before extraction. The yield of *FS* after enzymic hydrolysis was always greater than that obtained after acid hydrolysis (Table 10).

Table 10. *Comparison between the yield of FS obtained after β-glucuronidase and* pH 1 *hydrolysis*

(Samples (*c*), (*d*), (*e*), etc., are different 24 hr. samples from the same individual.)

	FS (mg. compound E/24 hr.)		
Urines	pH 1 hydrolysis for 24 hr. (*a*)	β-Glucuronidase hydrolysis (*b*)	Ratio (*b*/*a*)
J.H. (*c*)	3·1	12·6	4
(*d*)	1·0	3·9	3·9
(*e*)	2·6	15·0	5·8
(*f*)	2·4	19·5	8·1
(*g*)	1·9	9·3	4·9
(*i*)	1·2	15·4	12·8
V.C. (*a*)	0·8	2·1	2·5
(*b*)	0·5	13·6	27·2
(*c*)	0·8	9·7	12·1
(*d*)	2·4	15·7	6·5
E.E. (*a*)	2·5	19·5	7·8
(*b*)	0·8	11·5	14·4
(*c*)	1·0	9·1	9·1
(*h*)	0·7	5·8	8·3
(*i*)	1·6	3·1	1·9

There were wide fluctuations in the quantities of *FS* liberated by β-glucuronidase, and, although in general they tended to rise and fall in parallel with the values obtained after acid hydrolysis, there does not appear to be any relationship between the amount of steroid excreted as glucuronide and the amount obtained after acid hydrolysis.

DISCUSSION

Assuming that the formaldehydogenic substances measured by the method used are in fact formaldehydogenic steroids, the findings reported here and the observations of Heard & Sobel (1946) and of Marrian and his colleagues emphasize that corticosteroids are excreted in the urine in the following forms: (1) As free compounds which can be extracted from urine at pH 7·4. Some of these free compounds may be destroyed on prolonged contact with acid, but acidification to pH 1 followed by immediate extraction does not cause any significant loss. (2) As chloroform- or ether-soluble conjugates, some of which appear to be rapidly hydrolysed by acid to liberate a free acid-labile compound. Others, however, are not so rapidly hydrolysed by acid; they pass from acidified urine into the chloroform or ether phase whence they are extracted by alkali and can be hydrolysed at room temperature or 37° at pH 3–4·5. (3) As a conjugate which is slowly hydrolysed at room temperature and pH 1. (4) As a conjugate with glucuronide, which can be hydrolysed with β-glucuronidase. The free compounds liberated appear to be acid-labile. The proportion of these different forms appears to vary from person to person and in the same person from day to day.

The current methods of hydrolysis are clearly inadequate. If the urine is extracted immediately after acidification to pH 1, only free steroids and those rapidly hydrolysed from the chloroform-soluble conjugate will be obtained. If the urine is extracted 24 hr. after acidification free steroid will be obtained from that conjugate which is slowly hydrolysed at pH 1 and room temperature, but there will be a greater or lesser destruction of those steroids which are extractable immediately after acidification but are acid-labile. In neither case will there be effective hydrolysis of the glucuronide conjugates. A true index of adrenocortical function may not be given unless all the different excretory products are extracted and the total steroid output determined. Acidification is an essential step in hydrolysing those conjugates which rapidly yield a free compound. Since these free compounds, and also those initially excreted as such, are acid-labile, acidification must be followed by immediate extraction. Other conjugates are hydrolysed more slowly and so the urine must be extracted again after prolonged acidification. Hydrolysis with β-glucuronidase also appears essential, and since the enzyme may be inactivated by small amounts of organic solvent it is more practical to carry out the enzymic hydrolysis before the acid hydrolysis. It is tentatively suggested that the following procedure may extract the corticosteroids and their conjugated compounds which have so far been recognized as being present in urine:

(*a*) A suitable sample of urine is brought to pH 4·5 and buffered with 1·0M-acetate buffer. β-Glucuronidase is added and the urine incubated for 48 hr. The enzyme will hydrolyse the glucuronide conjugates, and in addition the pH and temperature may also hydrolyse some of the chloroform-soluble conjugates.

(*b*) The urine is then extracted without altering the pH from 4·5. This will remove steroids excreted initially in the free form and those liberated from glucuronide conjugation. It will also extract any free steroid liberated from the chloroform-soluble conjugates.

(*c*) The urine is adjusted to pH 1 and extracted immediately. This will remove that moiety which it has been shown is extractable immediately after acidification and which may arise from the rapid hydrolysis of any remaining chloroform-soluble conjugates.

(*d*) The urine is left at room temperature and pH 1 for 24 hr. before it is finally extracted. This will liberate free compounds from conjugates which are slowly hydrolysed by acid.

Preliminary trials using this technique have yielded 15–30mg. formaldehydogenic steroid/24hr., the largest moiety always being liberated from glucuronide conjugation. All the steroid obtained by β-glucuronidase hydrolysis would not necessarily be expected to be biologically active, and Venning (1951) has found that whereas glucuronidase hydrolysis may increase the yield of formaldehydogenic substances tenfold, the yield of glycogen-depositing steroids is only increased fivefold.

SUMMARY

1. Experimental data are presented which suggest that formaldehydogenic substances (*FS*, assumed to be corticosteroids) are excreted in urine as (*a*) free compounds, (*b*) in conjugation with glucuronic acid and (*c*) as at least two other conjugates.

2. Methods for hydrolysing the conjugates and extracting the free compounds are described.

This work was mainly carried out in the Department of Medicine, Presbyterian Hospital, New York City, during the tenure of a Rockefeller Travelling Fellowship in Medicine. I am most grateful to Dr Robert F. Loeb, Bard Professor of Medicine, for providing facilities and for his constant support. Miss Beatrice Silides gave invaluable assistance. I also wish to thank Dr Seymour Lieberman and Dr Joseph W. Jailer for their advice.

REFERENCES

Burton, R. B., Zaffaroni, A. & Keutman, E. H. (1951). *J. biol. Chem.* **188**, 763.
Callow, R. K (1950). *Hormone Assay*, p. 384, ed. by C. W. Emmens. New York: Academic Press.
Corcoran, A. C., Dustan, H. P. & Page, I. H. (1951) *J. clin. Invest.* **30**, 633.
Corcoran, A. C., Page, I. H. & Dustan, H. P. (1950). *J. Lab. clin. Med.* **37**, 297.
Cox, R. I. & Marrian, G. F. (1951). *Biochem. J.* **48**, xxxiii.
Daughaday, W. H., Jaffe, H. & Williams, R. H. (1948). *J. clin. Endocrinol.* **8**, 166.
Dorfman, R. I. (1950). *Hormone Assay*, p. 325 ed. by C. W. Emmens. New York: Academic Press.
Fishman, W. H. & Talalay, P. (1947). *Science*, **105**, 131.
Genest, J. (1951). Personal communication.
Heard, R. D. H. & Sobel, H. (1946). *J. biol. Chem.* **165**, 687.
Kinsella, R. A., Doisy, R. J. & Glick, J. H. (1950). *Fed. Proc.* **9**, 190.
Klyne, W. (1946). *Biochem. J.* **40**, iv.
Klyne, W. & Marrian, G. F. (1945). *Biochem. J.* **39**, xiv.
Lieberman, S. & Dobriner, K. (1948). *Recent Progress in Hormone Research*, II, p. 72. New York: Academic Press.
Lowenstein, B. E., Corcoran, A. C. & Page, I. H. (1946). *Endocrinology*, **39**, 82.
Mason, H. L. & Sprague, R. G. (1948). *J. biol. Chem.* **175**, 451.
Paterson, J. Y. F., Cox, R. I. & Marrian, G. F. (1950). *Biochem. J.* **46**, xxix.
Paterson, J. Y. F. & Marrian, G. F. (1951). *Biochem. J.* **48**, xxxiii.
Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.
Talbot, N. B., Saltzman, A. H., Wixom, R. L. & Wolfe, J. K. (1945). *J. biol. Chem.* **160**, 535.
Venning, E. H. (1951). Personal communication.
Venning, E. H., Kazmin, V. E. & Bell, J. C. (1946). *Endocrinology*, **38**, 79.
Zaffaroni, A., Burton, R. B. & Keutman, E. H. (1950). *Science*, **111**, 2871.

The Content of Glycogenolytic Factor in some Insulin Samples

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Sutherland & Cori (1948) have reported that a sample of insulin manufactured by the Eli Lilly Research Laboratories contained a glycogenolytic factor, whereas a sample prepared by the Novo Terapeutisk Laboratorium A/S contained none. Later work (Sutherland & Duve, 1948) has shown that the factor is secreted by the pancreas, probably by the α cells, and Sutherland & Cori have suggested that the factor is responsible for the initial hyperglycaemia which may follow an intravenous injection of insulin. The variations in this initial hyperglycaemia reported by different authors, e.g. Burger & Kramer (1930), suggest that the amount of factor associated with different insulin samples may vary. An assay of glycogenolytic-factor content has been carried out on some insulin samples available in this laboratory and has shown that the amount does vary considerably, both in samples prepared by different manufacturers and in samples from the same firm.

The test system was based on that described by Sutherland & Cori (1948) and Sutherland & Duve (1948), where activity was estimated from the increased rate of production of glucose by liver slices, incubated in buffered saline, caused by the addition of the factor. The experimental procedure is described somewhat fully, as satisfactory results were only obtained when it was followed in detail. Two methods of assay were used. The first, or single-dose, method gives an approximate estimate of activity after testing at one concentration; the second, or multiple-dose, method requires testing of samples at several concentrations and gives a more accurate assessment of relative potency.

EXPERIMENTAL

The test system

Reference standard. A sample of insulin containing the factor (Eli Lilly, no. 987267) was used as the reference standard for comparison of glycogenolytic-factor content.

Insulin samples. Samples, prepared by a number of manufacturers from the pancreas of ox, calf, sheep and pig, were given to us for assay by Prof. F. G. Young and Dr A. C. Chibnall.

Choice of animal. In preliminary experiments, liver slices from several animals were tested with the reference standard used at a concentration of 53 μg./ml., which was sufficient to give a maximum response with rabbit and rat liver (Table 1). While it would be an advantage to select an animal such as the cat, whose liver gives both a large basic output of glucose and a large response to the factor (especially when testing at concentrations of factor giving a sub-

maximal response), for routine testing a more readily available animal must be chosen and rabbits were used for all the assays reported here. The best results were obtained with liver from young (2–3 months) rabbits fed carrots, in addition to the stock pellet diet, for several days prior to use. Rabbits infected with coccidiosis were generally unsatisfactory and were not used except in very mild cases.

Table 1. *Species variation in response of liver slices to glycogenolytic factor*

(Control liver slices incubated in saline, test slices in saline containing the reference standard (53 μg./ml.). Incubation at 37° for 30 min.)

Animal	No. of livers tested	Mean glucose output (mg./g. liver): Control slices	Mean glucose output (mg./g. liver): Test slices
Guinea pig	3	5·6	6·6
Rat (hooded)	5	6·3	9·0
Rat (Wistar)	2	11·6	15·8
Rabbit	110	7·0	12·0
Ferret	2	15·1	20·6
Cat (adult)	1	23·1	31·1
Kitten	3	15·9	24·9

After the bulk of the assays had been completed a few experiments were carried out using Wistar rats, the results of which are included in Table 1. Liver slices from this strain produced more glucose than those from the hooded rats used in the preliminary experiments and it is possible that liver from these animals might provide a more consistent test system than rabbit liver, as more uniform animals would be available.

Incubation medium. Phosphate-saline, as recommended by Sutherland & Cori (1948), was used for incubation of the slices; it was prepared by diluting 1 vol. 0·1 M-sodium phosphate buffer (pH 7·4) with 4 vol. 0·9% (w/v) NaCl. Media containing the reference standard or the insulin samples to be tested were prepared immediately before use. The insulin was dissolved in 0·0033 N-HCl (0·5 ml./mg. insulin) and the solution was diluted with phosphate-saline to give the required concentration.

Preparation of liver slices. The rabbits were anaesthetized by injection of Nembutal and bled from the aorta. The liver was removed and chilled immediately by placing for 3–5 min. in ice-cold phosphate-saline. A small block of liver was cut into slices, 0·3–0·6 mm. thick and weighing about 200 mg., by means of a tissue slicer (Stadie & Riggs, 1944). The slices were placed on a chilled Petri dish and covered with a moist filter paper. Each slice was cut into several portions, not varying in weight by more than a few mg.; the portions were weighed on a torsion balance and dropped into 3 ml. chilled phosphate-saline, with or without insulin (see below), in 25 ml. conical flasks. (In early experiments test tubes were used, but the slices did not shake adequately in the medium.)

Incubation. The flasks were shaken at 110 oscillations/min. in a water bath at 37°. For the single-dose assays and most of the multiple-dose assays a 30 min. period was used, based on Sutherland & Cori's (1948) results for rat liver. Later it was found that in our conditions 60 min. incubation gave better results both for rabbit and rat (Wistar) liver and this period was used for one of the assays carried out by the multiple-dose method.

Glucose estimation. At the end of the incubation period the flasks were removed from the bath and cooled in ice. Portions of medium (2 ml.) were removed, deproteinized by addition of 1 ml. each of 0·3 N-$Ba(OH)_2$ and 5% (w/v) $ZnSO_4$, and glucose estimated by Somogyi's (1945) modification of the method of Nelson (1944).

Single-dose assay method

The concentration of reference standard, 53 μg./ml., was chosen to give a maximum response in the test system. Insulin samples were first assayed at the same concentration and those samples which showed little or no activity were retested at higher concentrations. No samples tested showed a higher activity than the reference insulin. In early experiments, liver slices were cut in two portions, one being used to determine the basic rate of glucose output (G), the other for the rate of glucose output in the presence of the standard (S) or of the sample (T). The percentage increase in glucose output was calculated for each slice,

$$100(S \text{ (or } T) - G)/G,$$

and the activity of the sample was expressed as 100 times the ratio of the mean percentage increase for the sample to that for the standard. In later experiments, liver slices were cut into three or four portions and the rates of glucose output

Table 2. *Glycogenolytic-factor content of insulin sample B 1 assayed by the single-dose method on several rabbits*

(For experimental details, see 'single-dose assay method'.)

Rabbit no.	Increase in glucose output over control (%): Individual slices: Standard	Individual slices: Sample	Mean for each rabbit: Standard	Mean for each rabbit: Sample	Activity of sample (Reference standard = 100)
15	95, 83, 64, 64	42, 40, 27, 21	77	33	43
17	63, 73, 77, 84	12, 30, 38, 19	74	25	34
20	138, 151, 152, 217, 282	30, 39, 71	188	47	25
21	72, 75, 83, 85	56, 26, 22, 10, 24	79	28	35
22	94, 107, 130	65, 25, 32, 25	110	37	34
24	47, 89, 94, 71	6, 4, 29, 10	75	12	16
26	59, 45, 33, 31, 48	18, 27, 15, 35	43	24	56
27	70, 30, 75, 72, 40	12, 33, 19, 37	57	25	44
				Mean	36

both under basal conditions and in presence of the standard and sample (or samples) determined on portions of the same slice.

In our experience variation between rabbits is such that several animals should be used to obtain a reasonable assessment of activity. Possibly if an adequate number of rabbits from the same strain had been available, variation would have been less. The activity reported is the mean for all the rabbits used. Results on one sample (Table 2) illustrate the range of variation for slices both from the same and different animals.

Table 3. *Glycogenolytic-factor content of insulin samples*

(Abbreviations used: *B*, Boots Pure Drug Co. Ltd.; *BW*, Burroughs Wellcome Ltd.; *K*, Kry; *N*, Novo Terapeutisk Laboratorium A/S, Denmark; *AH*, Allen and Hanburys Ltd.; *BDH*, British Drug Houses Ltd.; *BP*, Blatherwick Process; *X*, manufacturer unknown. Manufacturers identification number, when known, shown in parenthesis.)

Sample no.	Species	Single-dose method: No. of livers used for assay	Single-dose method: Activity (Reference standard = 100)
*B*1	Ox	8	36
*B*2	Ox	6	8
*B*3 (4840)	Ox	3	52
*B*4 (4840)	Ox	5	74
*B*5	Calf	2	29
*B*6	Sheep	1	26
*B*7 (3091)	Sheep	1	45
*B*8	Pig	2	15
*B*9	Pig	2	18
*B*10	Unknown	4	0
X	Pig	2	94
BP	Unknown	9	55
*BW*1 (9726)	Unknown	2	5
*BW*2 (9615)	Unknown	4	55
AH (2039)	Sheep	2	21
BDH (2065)	Pig	4	19
*K*1	Ox	10	64
*K*2	Pig	10	12
*N*1	Unknown	5	25
*N*2	Ox	2	5
*N*3	Calf	1	9
*N*4	Sheep	1	2
*N*5	Pig	2	1

Multiple-dose assay method

A number of samples and the standard were tested at four concentrations which were in geometrical progression. Pilot experiments were carried out to find the approximate activity of each sample, and concentration levels were chosen so that the responses of the standard and test samples were approximately the same at each of the four levels. In each experiment eight liver slices from the same rabbit were cut into three portions, a control portion being incubated in phosphate-saline without addition, while the other two were incubated in phosphate-saline containing either the standard or one of the samples under test at two concentrations. The design as a whole was a randomized block type, each slice being used for testing one sample at two concentrations. The logarithms of the relative potencies of the samples were calculated from the regressions of the responses on the logarithms of the concentrations.

RESULTS

Twenty-three samples of insulin were tested by the single-dose method (Table 3) at a concentration of 53 μg./ml., the same as that used for the standard. Eight which gave low values were retested at higher concentrations (Table 4); of these four proved to contain no glycogenolytic factor.

Table 4. *Glycogenolytic-factor content of insulin samples tested at high concentrations*

(Single-dose assay method. 30 min. incubation at 37°. Number of observations shown in parenthesis.)

Sample no.	Increase of glucose output over control (mg. glucose/g. liver): Standard (53 μg./ml.)	Sample (200 μg./ml.)
*N*2	4·08 (2)	0 (2)
*N*3		0 (2)
*N*4		1·36 (1)
*B*2		0·06 (1)
*N*5	4·48 (3)	0 (2)
*BW*1	4·50 (3)	1·74 (1)
*B*10	3·55 (10)	0·43 (6)
*K*2	3·56 (4)	0·40* (4)

* Sample *K*2 tested at 105 μg./ml.

Eleven of the samples were also assayed by the multiple-dose method, three simultaneously with the standard, in assay *A*, the others in a series of separate assays (*C*) against the standard (Table 5).

In assay *A*, the standard was assayed at 2, 4, 8 and 16 μg./ml. samples *B*5 and *N*1 at 20, 40, 80 and 160 μg./ml., and sample *X* at 3, 6, 12 and 24 μg./ml. Four experiments were carried out; in any one experiment each sample and the standard were tested at the lowest and highest concentrations on portions of one liver slice and at the two intermediate concentrations on portions of another slice. In this assay the slopes of the regression lines for all the samples and for the standard were not significantly different so that it is permissible to use the overall slope for calculation of relative potency.

The remaining samples (series *C*) were assayed during the development of this method. Statistical analysis of the results showed that the experiments only permitted the assay of each sample against the standard. However, the experiments treated in this way were well balanced, as each sample was tested simultaneously with the reference standard, at several concentrations, on liver slices from the same rabbit, and the results (except for one sample where the slope of the regression line was significantly

Table 5. *Relative glycogenolytic-factor potencies of insulin samples assayed by the multiple-dose method*

(For explanation of sample numbers see Table 3.)

	Assay *A*			Assay *C*		
Sample	Log_{10} relative potency (M)	Standard error of M (σ_M)	Relative potency (standard = 1) (10^M)	Log_{10} relative potency (M)	Standard error of M (σ_M)	Relative potency (standard = 1) (10^M)
*B*1	—	—	—	−1·0509	0·1723	0·09
*B*2	—	—	—	−4·4946	3·6592	0·003
*B*3	—	—	—	−0·6777	0·1141	0·21
*B*4	—	—	—	0·0139	0·0976	1·03
*B*4	—	—	—	−0·0293	0·1641	0·94
*B*5	−1·2005	0·0632	0·06	—	—	—
*B*7	—	—	—	−1·0091	0·5921	0·10
*B*9	—	—	—	−1·9392	0·8844	0·01
X	−0·3944	0·0632	0·40	—	—	—
BP	—	—	—	−0·7331	0·1943	0·18
*K*1	—	—	—	−0·6825	0·1721	0·21
*N*1	−1·3630	0·0632	0·04	—	—	—

different from that of the standard) are included in Table 5. One sample, *B*4, was assayed twice in this series of experiments and also in assay *B* (see below).

The multiple-dose assay method was also used (assay *B*) for testing insulin sample *B*4 and some fractions prepared from it by Dr G. R. Tristram. As the results give a further example of the use of the method they are reported in Table 6.

Table 6. *Assay of insulin sample B4, and of six fractions prepared from it, for glycogenolytic factor by the multiple-dose method*

Fraction no.	Log_{10} relative potency (M)	Standard error of M (σ_M)	Relative potency (10^M)
1	−0·6800	0·1234	0·21
2	−0·6344	0·1164	0·23
3	−2·2611	0·1415	0·005
4	−0·4265	0·1156	0·37
5	−1·5103	0·1135	0·03
6	−0·1992	0·1013	0·63
*B*4	−0·1262	0·1007	0·75

The statistical treatment of these assays is further considered by Mr N. W. Please in an Appendix to this paper. In assay *C*, another three fractions prepared by Dr G. R. Tristram (not shown in Tables 5 or 6) were tested, making up the total of thirteen test samples mentioned in the Addendum.

DISCUSSION

The single-dose method of assay can be carried out rapidly and gives a fair approximation of relative activity except for samples of very low or high glycogenolytic-factor content. In our experience the method suffers from two difficulties. Not only does the increase in glucose output due to the addition of either standard or test sample vary with individual liver slices from the same rabbit, but the apparent activity of the test substance varies from rabbit to rabbit. Variation in glucose output from parts of a single slice was less than between different slices so that the technique would be improved by always assaying the standard and test sample on portions of the same slice.

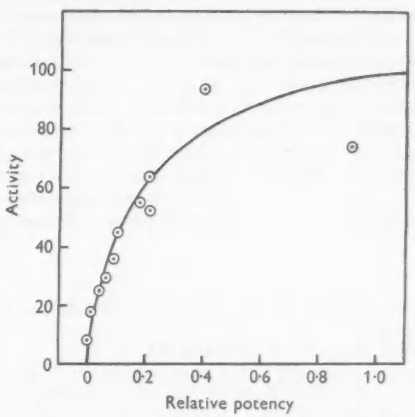

Fig. 1. Glycogenolytic activity of insulin samples. Comparison of activity (single-dose method, standard = 100) and relative potency (multiple-dose method, standard = 1·0).

A more accurate assay of relative potency is obtained if samples and standard are tested at several concentrations, arranged so that the responses to samples and standard are of the same order, and the results examined statistically.

After most of our assays had been carried out, Sutherland, Cori, Haynes & Olsen (1949) described a modification of their original method in which

samples were tested at three or more concentrations, usually on the liver from only one rabbit. As we have pointed out, in our experience it is better to use liver from more than one rabbit for accurate results and we think statistical analysis is advisable as an indication of the degree of accuracy.

The numerical values for the activity of samples assayed by the two methods would not be expected to be the same, since in the single-dose method activities are compared at the same concentration of sample, but not of glycogenolytic factor, whereas in the multiple-dose method relative potency is assessed by comparison of concentrations giving similar responses. However, for samples tested by both methods, except for two with high activity, *X* and *B* 4, there is a relation between the activity assayed by the single-dose method and the relative potency assessed by the multiple-dose method (Fig. 1).

Considerable variation was found in the amount of factor associated with the insulin samples tested. Samples prepared by one firm may contain different amounts of factor, e.g. of the ten samples from Boots Pure Drug Co. Ltd., two contained little or no factor, while others contained an appreciable amount. Two samples from this firm bore the same batch number (4840); of these, *B* 3 (amorphous), received in 1948, contained only a small amount of factor whereas *B* 4 (crystalline), received in 1949, contained about as much factor as the standard sample prepared by Eli Lilly. The reference standard (received in 1947) was the only sample prepared by Eli Lilly which we tested, but Sutherland *et al.* (1949) have since tested Lilly samples which were free from factor. One of the samples (*N* 1) prepared by Novo Terapeutisk Laboratorium contained an appreciable amount of factor, although the other four Novo samples tested were inactive. Two other samples (*K* 2 and *BW* 1), besides two of the Boots samples, were also free from factor.

This variability of samples prepared by the same firm from pancreas from the same species makes difficult the comparison of samples from the pancreas of different animals, but the results suggest there is not much difference in the factor content of pancreas of ox, sheep and pig. We had an opportunity of testing some insulin-inactive fractions, discarded during the manufacture of insulin by Boots, but none of them showed a higher factor content than the standard insulin.

SUMMARY

1. Two methods have been used for the assay of glycogenolytic factor in insulin samples. The first, or single-dose method, allows rapid testing of samples and gives an approximate estimate of activity; the second, or multiple-dose method, requires testing at several concentrations and statistical treatment of results.

2. Twenty-three commercial insulin samples have been assayed. The glycogenolytic-factor content was found to vary considerably; no samples were found to have a higher activity than the sample used as a standard (prepared by Eli Lilly and sent to us in 1947). Several were free from glycogenolytic factor.

We should like to express our thanks to Prof. F. G. Young, F.R.S., for his interest in this work and to him and to Dr A. C. Chibnall, F.R.S., for sending us samples of insulin for testing, as well as to the firms who supplied the samples. One of us (G.A.) is indebted to the Diabetic Association for the award of a Lund Fellowship.

REFERENCES

Burger, M. & Kramer, H. (1930). *Arch. exp. Path. Pharmak.* **156**, 1.

Nelson, N. (1944). *J. biol. Chem.* **153**, 375.

Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.

Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **154**, 687.

Sutherland, E. W. & Cori, C. F. (1948). *J. biol. Chem.* **172**, 737.

Sutherland, E. W., Cori, C. F., Haynes, R. & Olsen, N. S. (1949). *J. biol. Chem.* **180**, 825.

Sutherland, E. W. & Duve, C. de (1948). *J. biol. Chem.* **175**, 663.

APPENDIX

Statistical Examination of Multiple-dose Assays

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Assuming that, over the range of concentrations considered, the relation between response and the logarithm of the concentration is linear then, for a valid assay of a test substance against the standard, the two sets of points must lie on two parallel straight lines (Finney, 1947). Now the relative potency (R) of the unknown to the standard is the ratio of the concentrations of the standard and the unknown required to give the same response. Thus the logarithm of the relative potency (M) is the horizontal distance between the two lines and, since the lines are parallel, is independent of the level of response.

While a 'four-point' assay, in which the standard and the test substance are each used at two concentrations, would be sufficient to estimate M, it was decided to use each at four concentrations in order to test the assumptions of linearity and parallelism.

Assay A

The eight slices from a rabbit liver were allotted in random order to the standard and the three samples. Each insulin sample was used at four concentrations, which were in geometrical progression, the highest and lowest on one slice and the intermediate concentrations on another. An analysis of variance was carried out on the data from four rabbits (Table 1), the degrees of freedom for 'concentrations' being subdivided. Since responses were obtained at four concentrations, the logarithms of the concentrations being in arithmetical progression, the average response and linear, quadratic and cubic components were easily estimated for each insulin from the following formulae (Emmens, 1948):

$$\begin{aligned}\text{mean response} &= (Y_1+Y_2+Y_3+Y_4)/4,\\ \text{linear} &= (-3Y_1-Y_2+Y_3+3Y_4)/10,\\ \text{quadratic} &= (Y_1-Y_2-Y_3+Y_4)/4,\\ \text{cubic} &= (-Y_1+3Y_2-3Y_3+Y_4)/6,\end{aligned}$$

where Y_1 is the mean response (mg./100 g. liver) at the lowest concentration and so on.

It will be noted that comparisons between insulins were independent of differences between rabbits and, for each rabbit, estimates of linear and cubic components were unaffected by differences in slices, although such differences would affect the quadratic component.

Since the quadratic and cubic components were not significant it is reasonable to assume the relations were linear. Also, since the interaction insulins × linear was not significant it may be assumed that the slope of the linear relation (regression line) was the same for all four substances, the mean slope being 511 ± 34.

The logarithm of the relative potency (M) of each sample to the standard and the variance of M were calculated from the formulae (Emmens, 1948)

$$M = \bar{x}_s - \bar{x}_u - (\bar{Y}_s - \bar{Y}_u)/b,$$

$$\sigma_M^2 = \frac{\sigma^2}{b^2}\left(\frac{1}{n_s}+\frac{1}{n_u}\right) + \frac{(\bar{Y}_s-\bar{Y}_u)^2}{b^4}\,\sigma_b^2,$$

Table 1. *Statistical analysis of assay A*

Source	Sums of squares	Degrees of freedom	Mean square	Significance (%)
Rabbits	169 679	3	56 560	1
Insulins	296 047	3	98 682	1
Doses	1 920 832	3	—	1
Mean linear 1 892 970	—	1	1 892 970	1
Mean quad. 27 806	—	1	27 806	—
Mean cubic 56	—	1	56	—
Rabbits × insulins	202 171	9	22 463	5
Insulins × doses	65 918	9	—	—
Insulins × linear 4741	—	3	1 580	—
Insulins × quad. 58 984	—	3	19 661	—
Insulins × cubic 2193	—	3	731	—
Rabbits × doses	87 815	9	9 757	—
Residual	225 438	27	8 349	—
Total	2 967 900			

where $\bar{x}_s$ is mean log. concentration of the standard,
$\bar{Y}_s$ is mean response of the standard,
b is mean slope,
σ^2 is residual variance (8349·5),
n_s is number of responses for the standard (1b),
σ_b^2 is variance of slope (a function of σ^2).

The subscript u denotes the sample (or unknown). The values of M and σ_M are given in Table 5 of the main paper.

Assay B

The second series of experiments were arranged in a similar way except that there were seven samples and the standard. In this case it was necessary to confound the quadratic component with rabbit differences instead of slice differences. Thus the eight slices from each rabbit were allotted at random to the fractions and the standard and, in four of the rabbits, the highest and lowest concentrations were used on each slice while in the other four the two intermediate concentrations were used. The analysis (Table 2) was carried out in a similar way except that the quadratic component was tested against the 'between rabbits' mean square instead of the residual. Since neither the quadratic and cubic components nor the differences in the slopes were significant, the mean slope was used to calculate M and σ_M as in the previous experiment (Table 6 of main paper).

Table 2. *Statistical analysis of assay B*

Source	Sums of squares	Degrees of freedom	Mean square	Significance (%)
Insulins	1 034 508	7	147 787	1
Between rabbits (within sets of 4)	399 490	6	66 582	1
Mean linear	1 129 632	1	1 129 632	1
Mean quadratic (confounded with rabbits)	56 616	1	56 616	—
Mean cubic	5 029	1	5 029	—
Insulins × linear	110 690	7	15 813	—
Insulins × quad. (confounded)	131 838	7	18 834	—
Insulins × cubic	50 281	7	7 183	—
Insulins × rabbits (within sets of 4)	933 294	42	22 221	1
Residual error	363 294	48	7 565	—
Total	4 214 492	127	—	—

Assays C

The experiments in this series were, in fact, carried out before the others and were not planned in the same way, being pilot experiments. Since every insulin was used several times at several concentrations it was possible to calculate a residual variance for each substance by pooling the variances from each concentration. For each of the standard and the thirteen samples the linearity of the response-log. concentration relation was tested by comparing the deviations of response means at different concentrations from the regression line (fitted by the method of least squares) with the residual. In only one case was there a significant departure from linearity and this may probably be disregarded since a 5 % significant value may be expected by chance in twenty tests.

The residual variances were then combined and used to test whether the differences in the slopes were significant. They were found to be so at the 5 % level and it was therefore considered unsatisfactory to use a single mean slope for the calculation of relative potencies. These differences are probably due to one or two of the samples being on a different range of the response relation.

Since the experiment was arranged in eight assays, three with the standard and a single sample and five with the standard and two samples it was decided to examine each assay separately. For each assay the residual variances were pooled and used to test the differences of slope between samples and standard. In one case only was a significant difference obtained and this sample was discarded. In each of the other cases the assay mean slope was calculated, and hence the values of M and σ, from the formulae given (Table 5 of main paper).

The values of σ_M are rather large for two reasons. The repeated observations at each concentration were made on different rabbits and the residual variances are therefore inflated by differences between rabbits. The pooled residual variance was 10 876 compared with 8349 and 7565 in the other experiments. Also the mean responses of the samples and standard were often quite different thus giving a large factor

$$\frac{\sigma_b^2}{b^4}(\bar{Y}_s - \bar{Y}_u)^2$$

in the expression for σ_M^2. This was unavoidable since, these being pilot experiments, the relative potencies were unknown when the assays were started.

REFERENCES

Emmens, C. W. (1948). *Principles of Biological Assay.* London: Chapman & Hall.

Finney, D. J. (1947). *J.R. statist. Soc.* **9** B, 46.

The Content of Glycogenolytic Factor in Pancreas from Different Species

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Assays of glycogenolytic factor in samples of insulin prepared from the pancreas of ox, sheep and pig (Audy & Kerly, 1952) suggest that there is little difference in the glycogenolytic-factor content of pancreas from these animals. In an attempt to find a richer source of the factor, extracts of pancreas from cats, rats, ferrets and guinea pigs have been assayed for their glycogenolytic-factor content. Rabbit pancreas was also examined for comparison with the results of Sutherland & Duve (1948), who assayed the factor content of pancreas from rabbit, dog and ox. Since Sutherland & Duve (1948) and Heard, Lozinski, Stewart & Stewart (1948) have suggested that the factor is secreted by the α cells of the pancreas, assays were also carried out on extracts of tissue from the teleost fish *Lophius piscatorius*, a species in which islet tissue occurs separately from the pancreas proper, mostly in easily recognizable encapsulated structures (Mollendorf, 1939).

METHODS

Animals. All mammals were obtained from laboratory stock and were killed by injection of Nembutal. Three specimens of *L. piscatorius*, obtained from the Marine Biological Laboratory, Plymouth, were killed by a blow on the head. Pancreas was removed immediately after death and frozen in solid carbon dioxide. Microscopic examination of sections cut from the pancreas of *L. piscatorius* suggested that it contained acinar tissue only, while the encapsulated structures consisted mainly of islet tissue.

Preparation of extracts. Pancreas from individual cats and kittens was used for preparation of extracts; for the other species, pancreas from several animals was pooled. The frozen tissue was finely minced and extracts prepared as described by Sutherland & Duve (1948), except that the second acid-ethanol extraction was carried out for 2 hr. instead of 1 hr. Two ml. of final buffered extract corresponded to 1 g. pancreas, except for *L. piscatorius* where 10 ml. were equivalent to 1 g. of tissue. Trial extracts of rat pancreas, prepared by the acetone method of Kimball & Murlin (1923), showed little glycogenolytic-factor activity and the method was abandoned.

Determination of glycogenolytic-factor content. Activity was compared with the maximum activity of a standard sample of insulin containing the factor (Eli Lilly, no. 987267), using the test system already described (Audy & Kerly, 1952). In these tests the activities due to extract and standard were measured on portions cut from the same liver slice and, when possible, extracts were assayed on more than one rabbit for, as earlier recorded, we found some variation in activity when measured on liver slices from different animals. Activity was expressed, as suggested by Sutherland, Cori, Haynes & Olsen (1949), as the weight of pancreas required to produce 50% of maximum activity. This value was determined by testing extracts at several concentrations and plotting the activity (as % of the maximum activity) against the weight of pancreas equivalent to the amount of extract used. The method is illustrated in Fig. 1,

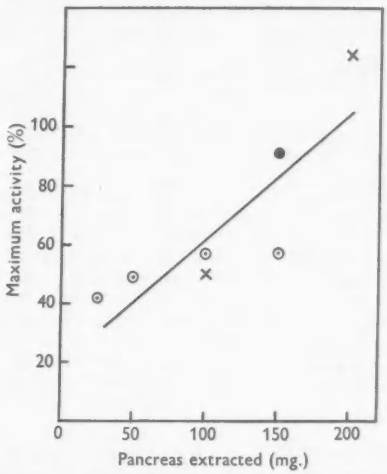

Fig. 1. Glycogenolytic-factor activity of an extract of rabbit pancreas tested on liver slices from three rabbits. ⊙, × and ● refer to the individual rabbit livers.

where the results of assaying an extract of rabbit pancreas on liver slices from three rabbits are shown. Since this method of expressing activity does not allow of ready comparison of the amount of factor in pancreas from different species, we have also calculated the 'relative activity' to be expected from 100 mg. pancreas, i.e. 100 divided by the weight of pancreas required to produce 50% of maximum activity.

RESULTS AND DISCUSSION

The results of the assays carried out are shown in Table 1. They are not directly comparable with those of Sutherland & Duve (1948) as the volume of our test system was larger, i.e. 3 ml. instead of 1·2 ml. However, if it be assumed that activity is proportional to the concentration of the factor

rather than to the total amount in the test system, then it can be calculated that the 'relative activity' of Sutherland & Duve's extracts would be: rabbit, 1·6; dog, 0·8; ox, 1·1; foetal calf, 8·0. The value for rabbit pancreas is similar in both assays, and all the extracts show the same order of activity. Cat pancreas contains somewhat more factor than that from the other species examined, but is not sufficiently active to make it a better source of factor than the more readily available ox pancreas.

Table 1. *Glycogenolytic-factor activity of pancreas*

(For experimental details and definition of relative activity, see Methods section.)

Animal	Weight of pancreas (mg.) giving 50% max. activity	Relative activity
Cat, adult	<25	>4
	50	2
Cat, kitten	<12	>8
(10–12 weeks)	<25	>4
	40	2·5
	<25	>4
Rat, hooded	50	2
	<50	>2
Rat, albino	225	0·4
	85	1·2
Rabbit, adult	67	1·5
Rabbit, 5 weeks	100	1
Guinea pig	100	1
	100	1
Ferret	150	0·7
Lophius piscatorius		
Islet tissue	60	1·7
Acinar tissue	(Very slight activity)	—

In contrast to the much greater activity shown by foetal calf pancreas as compared with ox pancreas in Sutherland & Duve's tests, we found little difference in activity of pancreatic extracts from young and adult animals (rabbit and cat). In view of this and of our previous observation (Audy & Kerly, 1952) that insulin samples prepared from calf and ox pancreas contain similar amounts of factor, it is suggested that the number of islet cells/unit weight of pancreas may decrease after birth in the ox, as in rat pancreas, where Hess & Root (1938) have reported a high ratio at birth which decreases rapidly as the young develop.

Further evidence for the production of glycogenolytic factor by the islet cells is provided by the results of assays of *L. piscatorius* tissue; extracts of islets contained an appreciable amount of factor, whereas those of acinar tissue contained only traces, which are possibly due to the presence of very small islets embedded in it (McCormick, 1924). Guinea pig pancreas is well known to have a high ratio of islet to acinar tissue, but extracts did not show a higher glycogenolytic-factor content than those from pancreas of other species examined. If the factor is secreted by the α cells the ratio of α to β cells would be of more significance in controlling the amount of factor secreted than the total amount of islet tissue but, so far as we are aware, no investigation of this ratio has been undertaken.

SUMMARY

1. The glycogenolytic-factor content of pancreatic extracts from rabbit, rat, cat, guinea pig and ferret has been assayed; all extracts gave similar values.

2. Extracts of islet tissue from *Lophius piscatorius* were active whilst those of acinar tissue were almost inactive.

We should like to thank the Director of the Marine Biological Association Laboratory, Plymouth, for assistance in obtaining specimens of *Lophius piscatorius* and the Eli Lilly Research Laboratories for the sample of insulin used as a reference standard. The thanks of one of us (G.A.) are also due to the Diabetic Association for the grant of a Lund Fellowship.

REFERENCES

Audy, G. & Kerly, M. (1952). *Biochem. J.* **52**, 70.
Heard, R. D. H., Lozinski, E., Stewart, L. & Stewart, R. D. (1948). *J. biol. Chem.* **172**, 857.
Hess, W. N. & Root, C. W. (1938). *Amer. J. Anat.* **63**, 489.
Kimball, C. P. & Murlin, J. R. (1923). *J. biol. Chem.* **58**, 337.
McCormick, N. A. (1924). *Trans. Canad. Inst.* **15**, 57.
Mollendorf, W. von (1939). *Handbuch der mikroskopischen Anatomie des Menschen*, 1st ed. Berlin: Julius Springer.
Sutherland, E. W., Cori, C. F., Haynes, R. & Olsen, N. S. (1949). *J. biol. Chem.* **180**, 825.
Sutherland, E. W. & Duve, C. de (1948). *J. biol. Chem.* **175**, 663.

The Effect of Varying Amounts of Dietary Cholesterol and of Choline upon Liver Lipids

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The early investigations of the effects of feeding cholesterol or foods rich in cholesterol have been reviewed by Duff (1935). The role of the lipotropic factors in protecting the liver against excessive deposits of lipids following the ingestion of cholesterol has been discussed by Best & Ridout (1939) and by McHenry & Patterson (1944).

In spite of the volume of work which has been carried out upon the effects on liver lipids of feeding cholesterol, no paper has been found which describes a systematic study of feeding graded doses of this substance. To obtain some information on the subject, twenty-one synthetic diets were prepared containing various amounts of cholesterol, up to 1·6 %. Six of the diets contained no choline. The lipotropic effect of supplementary choline chloride was noted at two dietary levels (equivalent to 0·08 and 0·32 % choline) and of inositol at 0·32 %. Even at the highest level of cholesterol intake, choline had a marked effect in preventing a rise in the glyceride fraction and exerted a considerable but less pronounced effect on the cholesteryl esters of the liver. The lipotropic action of inositol under these conditions was negligible.

Several rations typical of those consumed by average families in this country were analysed for cholesterol and choline. The values found for cholesterol are possibly slightly high since it is known that certain other sterols are precipitated by digitonin, and some of these give a colour with the Liebermann-Burchard reagent. Although cholesterol is found only in products of animal origin, many phytosterols and sitosterols possess such similar properties that 'apparent cholesterol' values are often obtained when foods of vegetable origin are analysed by the Schoenheimer-Sperry method. To that extent, estimations made on pooled food mixtures may be too high. The choline content of these rations appeared adequate to protect the livers of rats consuming such diets against excessive accumulation of glycerides or cholesteryl esters. If one assumes that these ordinary diets are not harmful to the livers of persons consuming them, one might be tempted to conclude that the choline requirement (as percentage of dry dietary ingredients) of the rat and of man is similar. It is obvious, however, that further work may reveal many discrepancies between the effects of cholesterol and choline upon the liver of the rat and of man.

EXPERIMENTAL

White rats (210) of the Wistar strain (80–120 g. in weight) were divided among twenty-one comparable groups. The animals, in individual cages fitted with a false bottom of coarse wire screen, were group pair-fed for 3 weeks. Weighed amounts of fresh food were supplied daily in feed trays designed to minimize spilling; left-over food and scatter were weighed the following morning. From these data the average daily individual food consumption was calculated. Fresh water was always available.

The basal diet (essentially free from choline) had the following percentage composition: casein (fat-free, vitamin-free) 8, gelatin 12, salt mixture no. 185 (McCollum & Simmonds, 1918) 5, cellu flour 2, 'vitamin powder' 1, sucrose 60, beef fat 10, corn oil 2 and cod-liver oil concentrate 0·015. The 'vitamin powder' consisted of thiamine hydrochloride 500 mg., riboflavin 250 mg., pyridoxine 200 mg., calcium pantothenate 1000 mg. and nicotinic acid 1000 mg. made to 1000 g. with very finely powdered (100 mesh) sucrose. Rats eating 10 g. of food per day receive 50 μg. thiamine hydrochloride and proportional amounts of the other vitamins daily. The cod-liver oil concentrate (Ayerst, McKenna and Harrison Ltd., Montreal), contained 200 000 i.u. vitamin A and 50 000 i.u. vitamin D/g.

Several groups of diets were prepared as shown in Table 1, containing five levels of crystalline cholesterol (British Drug Houses Ltd.) namely, 0·1, 0·2, 0·4, 0·8 and 1·6 %. The cholesterol was incorporated in these diets (as in similar fat-free ones) by grinding it in a mortar with small portions of the dry ingredients; this mixture was spread by means of a fine sieve over the main bulk of the dry mixture. The 'vitamin powder' and lipotropic supplements were treated similarly. Cod-liver oil concentrate dissolved in light petroleum was sprayed over the combined dry ingredients; after evaporation of the solvent the warm fats were poured over this dry mixture and blended thoroughly. Diets 6 to 11 contained 8 mg. of choline (supplied as the chloride in amounts 1·15 times the weight of the base) in 10 g. of diet, which was the anticipated daily food intake. This amount of choline was chosen since other experiments (Best, Lucas, Ridout & Patterson, 1950) had suggested that in rats on such a basal diet, 8 mg. of choline/10 g. of food are sufficient to maintain practically normal liver lipids. Similar rations with four times this amount of choline (diets 12 to 17) were fed to see whether the greater amount was sufficient to protect against the development of the so-called 'cholesterol fatty liver'. Diets 18 to 20 contained 0·32 % inositol.

After the animals had been fed the rations for 3 weeks they were stunned and their livers were removed immediately and weighed. The pooled livers from each group of ten rats were ground in a Waring Blendor with some acetone, extracted several times with hot absolute ethanol and the lipids in the extracts were taken up in light petroleum. The analytical details have been described previously by Best, Lucas, Patterson & Ridout (1946).

It is interesting to compare the dietary levels of cholesterol and of choline in these experimental diets with those in natural rations consumed by man. To obtain data on this latter point representative servings of all foods and beverages offered to the nurses of a local hospital were collected on three separate days through the courtesy of the dietitian. The mixture for each day was thoroughly blended and analysed for solids, ash, total lipids, free and total cholesterol, phospholipins (light petroleum-soluble $P \times 25$), choline (by microbiological assay with the cholineless mutant no. 34486 of the bread mould, *Neurospora crassa*) and inositol (with the Hansen no. 1 strain of *Saccharomyces cerevisiae* American Type Culture Collection, Washington D.C. (A.T.C.C.) 7921).

Table 1. *Effects of dietary cholesterol on rats*

(Diets fed for 3 weeks to groups of 6 F., 4 M.; 80–120 g.)

Group no.	Supplements (%)	Mean food intake (g./day)	Mean wt. change (%)	Liver: Mean wet wt. (g.)	Liver: Mean fat-free dry wt. (g.)	Composition of liver lipids (mg./liver): Total lipids	Cholesterol: Free	Cholesterol: Total	Cholesterol: Ester*	Glyceride
	Choline, 0									
0	Cholesterol 0	8·4	−5	6·0	0·991	1892	11·4	25·0	22·9	1700
1	0·1	8·9	−3	6·4	1·078	1829	12·0	34·4	37·9	1612
2	0·2	9·4	+5	7·5	1·176	2464	13·8	57·9	74·3	2219
3	0·4	9·7	+5	7·4	1·121	2716	15·0	100	143	2379
4	0·8	8·9	−4	8·6	1·138	3453	16·3	162	245	3008
5	1·6	8·8	−6	8·8	1·093	3929	18·6	208	320	3411
	Choline, 0·08									
6	Cholesterol 0	9·7	+10	4·5	1·024	390	9·6	12·8	5·4	224
7	0·1	9·7	+12	4·6	1·060	362	9·9	14·4	7·6	181
8	0·2	9·6	+16	5·0	1·064	549	11·6	32·0	34·3	338
9	0·4	9·8	+18	5·1	1·087	641	12·8	81·0	115	324
10	0·8	10·3	+22	5·8	1·222	795	14·8	126	187	414
11	1·6	9·8	+12	5·0	1·063	946	14·7	141	213	555
	Choline, 0·32									
12	Cholesterol 0	9·7	+22	4·6	1·088	351	10·1	12·5	4·0	174
13	0·1	9·5	+17	4·6	1·069	323	9·9	14·0	6·9	144
14	0·2	9·0	+12	4·7	1·067	321	10·4	18·0	12·8	125
15	0·4	9·9	+17	4·6	1·044	432	12·4	49·0	61·6	201
16	0·8	9·4	+14	4·7	1·050	477	13·8	64·6	85·6	210
17	1·6	10·1	+28	5·1	1·095	601	14·8	110	160	237
	Inositol, 0·32									
18	Cholesterol 0·1	9·5	+2	5·9	0·999	1754	11·1	35·6	41·2	1544
19	0·2	9·5	+4	5·7	0·972	1631	11·3	43·8	54·7	1413
20	0·4	9·9	+15	6·3	1·087	1815	13·8	88·5	126	1492

* Cholesteryl esters were calculated as oleate by multiplying bound cholesterol by 1·68.

RESULTS

The effects of the different intakes of cholesterol are presented in Table 1 as absolute weights (mg. per liver) of the lipid components, together with the average wet weights and the average fat-free, dry weights of the livers of each group so that each lipid component may be calculated to either basis. It is necessary under certain circumstances, as will be pointed out later, to compare the results expressed as percentage of the fat-free dry weight, in order to interpret the data properly.

Table 1 shows that in the liver lipids of the rats on the hypolipotropic basal diet glycerides have been doubled by the largest dose of cholesterol (1700–3411 mg.) and cholesteryl esters have increased 14-fold (23–320 mg.). Free cholesterol increased only slightly (11·4–18·6 mg.) and phospholipins, which are not shown in the table, remained essentially constant. When the results are expressed as percentage of wet weight, the rather large changes in the absolute values are less obvious, e.g. glyceride increased from 28·6 to 38·6 % of wet liver weight, cholesteryl esters from 0·39 to 3·6 %, free cholesterol from 0·19 to 0·21 %.

The effects of increasing amounts of dietary cholesterol upon total lipids and cholesteryl esters are plotted in Fig. 1 as percentage of wet weight. Both fractions were increased markedly by increasing dietary cholesterol when the rations were free from choline (groups 0–5; curves *A* and *A'*). The

presence of 0·08 % choline (groups 6–11; curves *B* and *B′*) as anticipated, greatly reduced the total lipids at each level of cholesterol intake but, unexpectedly, the cholesteryl esters appear to have been increased, e.g. cholesteryl oleate as percentage of wet weight is greater in group 11 (4·30 %) than in group 5 (3·62 %). A simple explanation for this apparently anomalous observation is presented later in the paper.

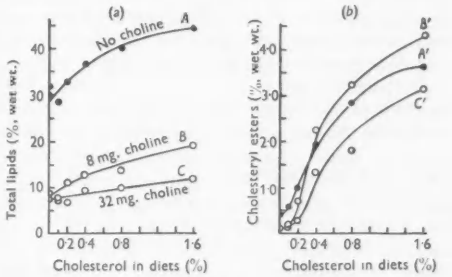

Fig. 1. Influence of dietary cholesterol on liver lipids. The curves on the left-hand side show the total lipids, as percentage of fresh liver; the curves on the right give the cholesteryl esters as percentage of fresh liver. Curves labelled *A* depict data from rats on diets without choline, curves labelled *B* show data from rats given diets containing 0·08 % choline and curves labelled *C* are drawn from data for rats on diets containing 0·32 % choline (supplied as chloride in amounts 1·15 times the values mentioned).

The data are plotted in Fig. 2 to show the lipotropic effect of increasing amounts of dietary choline in rations containing different amounts of cholesterol. Curves *A*, *A′* and *A″* refer to rats ingesting cholesterol at 1·6 % dosage, curves *B*, *B′* and *B″* to 0·8 % cholesterol, and curves *C*, *D*, *E* and *F* to 0·4, 0·2, 0·1 and 0 % respectively. The curves on the left-hand side illustrate the influence of choline upon the glyceride and cholesteryl ester fractions, in the basal ration and at two dietary levels of cholesterol intake, expressed in the usual way as percentage of wet weight of liver. It is obvious from the figure (curves *A″*, *B″* and *F″*) that the percentage of glycerides has been greatly reduced by 0·08 % of choline, but, as was noted previously, with the lower dose of choline an unexpected increase seems to have occurred in the bound cholesterol (curves *A′* and *B′*). In Table 1 the actual reduction in weight of the glyceride fraction can be seen to be from 3411 to 555 mg. (groups 5 and 11). Largely due to this removal of glyceride there is a reduction in weight of the liver from 8·8 to 5·0 g. Although the smaller dose (0·08 %) of choline reduced the weight of cholesteryl esters from 320 to 213 mg. per liver, the values expressed as percentage of wet weight have increased (from 3·62 to 4·26 %). When the results are expressed as percentage of fat-free, dry weight (see curves at right-hand side of Fig. 2) or as absolute weight, the lipotropic effect of choline upon the cholesteryl esters is clear.

For the benefit of those unfamiliar with normal values for liver lipids when expressed as percentage of dry fat-free tissue, the following data for ten rats from our stock colony may be reported: total lipid 20–32 % (av. 25 %), glyceride 9–18 % (av. 12 %),

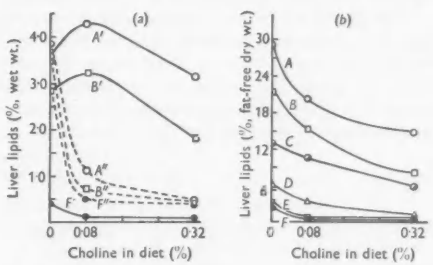

Fig. 2. Influence of dietary choline on liver lipids of rats ingesting different amounts of cholesterol. Cholesteryl esters are shown as solid lines, glycerides × 1/10 as broken lines. Data shown on the left-hand side are expressed as percentage of fresh liver weight, on the right-hand side as percentage of fat-free, dry liver. Curves *A*, *A′* and *A″* refer to diets containing 1·6 % cholesterol, *B* to 0·8 %, *C* to 0·4 %, *D* to 0·2 %, *E* to 0·1 % and *F* to an essentially cholesterol-free diet.

total cholesterol 0·9–1·2 % (av. 1·0 %), free cholesterol 0·7–1·0 % (av. 0·87 %), cholesteryl esters (calculated as oleate) 0·18–0·52 % (av. 0·26 %) and phospholipins 10·2–12·5 % (av. 11·8 %).

Analysts are well aware of the different impressions that one may obtain from different methods of expressing analytical findings. Biochemists and physiologists are less conscious of the erroneous notions that can arise if data are expressed as percentage when some component (not considered or mentioned because it is not one of interest at the moment) undergoes a large alteration simultaneously with the one being followed. The two methods of presenting the data for cholesteryl esters shown in Fig. 2 illustrate how certain statements in the literature may be wrong, due to neglect to consider the effect upon cholesteryl esters of a simultaneous large decrease in the amount of glycerides in the liver.

Channon & Wilkinson (1935) were puzzled by the fact that increasing the casein content of a hypolipotropic diet (containing 20 % fat and 2 % cholesterol) from 5 to 30 % caused an increase in cholesteryl oleate. The data of Beeston, Channon & Wilkinson (1935) show a similar effect. The explanation given above probably applies; in their published data a large decrease in glycerides may be seen to

have occurred, but since no absolute values are given it is impossible to verify the suggestion. The increase which some workers have noted in phospholipin under the influence of choline has a similar explanation. Actually, the weight of phospholipin in mg. per liver (or better, percentage of fat-free, dry liver tissue if the rats are not of uniform weight) remains essentially constant, but as glycerides are lost and the liver weight decreases, the value expressed as percentage of wet weight increases.

Thus, it should be emphasized that whenever a lipotropic substance produces a great decrease in the amount of glycerides in the liver, an erroneous impression may be gained of the nature of the changes in other lipid components if the values are expressed as percentage of wet liver weight.

done several years ago, that there is a sex difference in the response of rats to hypolipotropic diets, and that consequently there is an apparent difference between males and females in the lipotropic effect of inositol (Best, Ridout, Patterson & Lucas, 1951). Further, the livers were pooled before analysis, and with no knowledge as to the variability of the results it is impossible to estimate the significance of the relatively small differences observed when inositol was added to the diets. A reliable interpretation of the data can be made under these circumstances only when large and consistent differences are found, as was the situation with choline.

The cholesterol and choline values found by analysis of the nurses' diets, with other pertinent analytical data, are shown in Table 2. It may be

Table 2. *Lipid components of some human rations*

(Amounts ingested, g./day.)

Sample	Total lipid	Cholesterol			Phospholipin	Choline	Inositol
		Total	Free	Oleate			
1	87	0·625	0·513	0·189	5·78	0·305	1·04
2	96	1·125	1·013	0·189	7·51	0·534	1·16
3	83	0·400	0·313	0·147	2·89	0·267	0·49
Mean	**89**	0·717	0·613	0·175	5·39	0·369	0·90
		Expressed as percentage of dry solids in diet					
1	19·1	0·137	0·112	0·041	1·27	0·067	0·23
2	19·1	0·223	0·202	0·038	1·49	0·106	0·23
3	17·5	0·085	0·066	0·031	0·61	0·057	0·10
Mean	**18·6**	0·148	0·127	0·037	1·12	0·077	0·19

The rats in these experiments were fed the diets for only 3 weeks, but in other studies previously reported similar diets containing 0·5 % cholesterol were fed for periods of 8 and 16 weeks (Ridout, Lucas, Patterson & Best, 1946). Glycerides in the liver were maintained at normal levels by choline throughout the experiment, and a marked lipotropic effect on the cholesteryl esters was still demonstrable even if they were distinctly above normal after the rats had been on the rations for 4 months (4·5 % without choline, 1·0 % with choline, 0·05 % being normal).

Groups 18 to 20 illustrate the effects of increasing amounts of cholesterol (0·1, 0·2 and 0·4 %) in diets liberally supplied with inositol (0·32 %). Total lipids remained essentially unchanged at 29–30 % of wet liver weight. No great decrease in lipids due to the presence of inositol, such as occurred with choline, is to be noted, e.g. 0·4 % of dietary cholesterol in the basal hypolipotropic diet produced total liver lipids of 36·5 %, while inositol reduced this to only 28·7 % (groups 3 and 20). No significant decrease in cholesteryl esters was observed, but the glyceride fraction did seem to be decreased (compare 2379 mg. with 1492 mg. per liver). Unfortunately, it was not recognized, when these experiments were recorded also that the total wet weights (including beverages) were 2980, 3020 and 3035 g. (mean 3012 g.) of which the total solids amounted to 456, 504 and 472 g., respectively (mean 477 g.). The ash contents of these diets were found to be 11·1, 11·6 and 10·6 (mean 11·1 g.). The data in Table 2 are first presented as grams consumed daily and then in the more useful form (for comparative purposes) of percentage in the dry solids.

DISCUSSION

These results require but little discussion. It might be mentioned that the cholesterol-rich diets containing liver or egg yolk fed by Blatherwick, Medlar, Bradshaw, Post & Sawyer (1931, 1932, 1933*a*, *b*) and by Okey, Yokela & Knock (1933–34) and Okey & Yokela (1936), contained quantities of lecithin providing amounts of choline considerably outside the range examined in the present study. It is therefore impossible to make exact comparisons, but it is obvious from Fig. 2 that the slope of the curves relating liver cholesteryl esters to dietary choline is rapidly decreasing as choline increases. Thus considerably larger doses of choline, while exerting a marked lipotropic effect, would

probably leave the cholesteryl esters much above normal.

The original experiments of Best and his colleagues on the effects of lecithin and of choline were conducted with high-fat diets devoid of cholesterol: the excessive deposition of liver lipids was readily prevented under these conditions.

As shown in Fig. 2 (curves F' and F'') the large accumulation of glycerides (28·6 %) and the moderate increase in cholesteryl esters (0·39 %) that resulted from feeding the basal hypolipotropic diet free from cholesterol are both completely prevented by choline. The ready response of both fractions to the lipotropic influence of choline in this situation (i.e. the so-called 'fat fatty liver') may be contrasted with that in the case of the 'cholesterol fatty liver' in which choline, although it has almost as marked an effect on the glycerides (curves A'' and B''), inhibits only partially the deposition of cholesteryl esters at the higher intakes of cholesterol (cf. curves A' and B', or better, curves A to F in Fig. 2).

The average choline content (0·077 %) of the nurses' diets was found by coincidence to correspond almost exactly with the lower level of choline (0·08 %) used in the experiments reported above. It must be remembered that natural diets contain choline precursors (methionine, betaine and possibly thetins) besides the choline which can be estimated microchemically or microbiologically. Mere knowledge of analytical data for these substances does not permit an estimate to be made of what proportion is actually used for lipotropic purposes, but undoubtedly some is so used and supplements the dietary choline. While it is dangerous to assume that data obtained from rats can be applied to man, it is interesting to note that the average percentage of choline found in the diets of the nurses is just about that required to maintain normal levels of glycerides and cholesteryl esters in the livers of rats consuming diets containing proportions of cholesterol similar to those found in the nurses' rations.

SUMMARY

1. Crystalline cholesterol was incorporated at five concentrations between 0·1 and 1·6 % in hypolipotropic diets and in two similar sets of diets containing 0·092 and 0·368 %, respectively, of choline chloride. Three diets were prepared containing different levels of cholesterol with 0·32 % inositol. Liver lipids were determined after these rations had been fed to rats for 3 weeks.

2. A large deposition of glycerides and some increase in cholesteryl esters occurred in the livers of the rats consuming the basal hypolipotropic diet free from cholesterol.

3. Increasing amounts of dietary cholesterol caused a progressive increase in the accumulation of both glycerides and cholesteryl esters in the livers.

4. The larger amount of choline was able to maintain liver glycerides within or only slightly above the normal range at all these intakes of cholesterol.

5. Cholesteryl esters in the liver were slightly elevated even at the larger intake of choline when the diet contained 0·2 % of cholesterol; further increases in dietary cholesterol caused progressive increases in the deposition of cholesteryl esters.

6. Data are given for the lipid components (including cholesterol and choline) of several human diets.

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REFERENCES

Beeston, A. W., Channon, H. J. & Wilkinson, H. (1935). *Biochem. J.* **29**, 350.

Best, C. H., Lucas, C. C., Patterson, J. M. & Ridout, J. H. (1946). *Biochem. J.* **40**, 368.

Best, C. H., Lucas, C. C., Ridout, J. H. & Patterson, J. M. (1950). *J. biol. Chem.* **186**, 317.

Best, C. H. & Ridout, J. H. (1939). *Ann. Rev. Biochem.* **8**, 349.

Best, C. H., Ridout, J. H., Patterson, J. M. & Lucas, C. C. (1951). *Biochem. J.* **48**, 448.

Blatherwick, N. R., Medlar, E. M., Bradshaw, P. J., Post, A. L. & Sawyer, S. D. (1931). *Proc. Soc. exp. Biol., N.Y.*, **29**, 345.

Blatherwick, N. R., Medlar, E. M., Bradshaw, P. J., Post, A. L. & Sawyer, S. D. (1932). *J. biol. Chem.* **97**, xxxiii.

Blatherwick, N. R., Medlar, E. M., Bradshaw, P. J., Post, A. L. & Sawyer, S. D. (1933*a*). *J. biol. Chem.* **100**, xviii.

Blatherwick, N. R., Medlar, E. M., Bradshaw, P. J., Post, A. L. & Sawyer, S. D. (1933*b*). *J. biol. Chem.* **103**, 93.

Channon, H. J. & Wilkinson, H. (1935). *Biochem. J.* **29**, 350.

Duff, G. L. (1935). *Arch. Path.* **20**, 81, 259.

McCollum, E. V. & Simmonds, N. (1918). *J. biol. Chem.* **33**, 63.

McHenry, E. W. & Patterson, J. M. (1944). *Physiol. Rev.* **24**, 128.

Okey, R. & Yokela, E. (1936). *J. Nutrit.* **11**, 463.

Okey, R., Yokela, E. & Knock, G. (1933–34). *Proc. Soc. exp. Biol., N.Y.*, **31**, 507.

Ridout, J. H., Lucas, C. C., Patterson, J. M. & Best, C. H. (1946). *Biochem. J.* **40**, 494.

The Analysis of Hair Keratin

1. APPLICATION OF MICROBIOLOGICAL TECHNIQUES TO HYDROLYSATES OF HUMAN HAIR

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Analytical difficulties encountered during attempts to determine amino-acids in (or isolate them from) protein hydrolysates containing a large proportion of cystine first interested one of us (C.C.L.) nearly twenty years ago, but because the study was carried on sporadically, as a side-line, it progressed only slowly (Lucas & Beveridge, 1940; Beveridge & Lucas, 1944*a–c*). The development since about 1943 of microbiological assay procedures for amino-acids has made obsolete the laborious techniques previously used in studying protein hydrolysates. To get more complete and more reliable analytical data on the composition of human hair these newer methods have been applied to a freshly prepared hydrolysate of the same sample of cleaned hair as had been used by Beveridge & Lucas. The hair had meanwhile been stored in a tightly stoppered (plastic screw-cap) brown bottle in a dark cupboard. New analytical data for moisture (8·13 %), ash (0·51 %), and nitrogen, on moisture- and ash-free keratin (16·4 %) agreed well with the earlier determinations. Approximate values were obtained for most of the amino-acids in human hair keratin (Table 1); for comparison the values found some years ago by chemical procedures are included.

METHODS

Most of the amino-acids were determined by microbiological assay techniques, essentially as described by Henderson & Snell (1948); these were applied directly to the hydrolysate without removal of any cystine. Alanine was estimated by the method of Sauberlich & Baumann (1949) and serine was determined according to Stokes & Gunness (1945). Five organisms (shown in Table 1) were employed to assay the amino-acids. Standards were set up at five levels and tests at four levels, with tubes in triplicate in each case. Tryptophan was estimated chemically on unhydrolysed keratin by the method of Spies & Chambers (1949) which involves condensation with *p*-dimethylaminobenzaldehyde in 19 N-H_2SO_4 and development of a blue colour upon treatment with $NaNO_2$. Cystine in the hydrolysate was estimated by Lugg's (1933) modification of Sullivan's (1926) procedure. The chemical methods used for the other amino-acids have been described in detail elsewhere (Beveridge & Lucas, 1944*a–c*), but the fourth column of Table 1 indicates the general nature of the procedures used in these determinations.

RESULTS AND DISCUSSION

Analytical values for nineteen of the commoner amino-acids in human hair, as determined in this laboratory (fourteen by chemical and sixteen by microbiological methods), are shown in Table 1. Those familiar with the fractionation of protein hydrolysates know that significant losses usually occur in the mother liquors during precipitation of copper and zinc salts and during recrystallization of the free amino-acids. The figures in column 5, Table 1, for alanine, the leucines and valine are therefore given in parentheses, with a plus sign, to indicate that while the chemical work established the presence of these amino-acids, the amounts isolated represent minimal values. It is unreasonable, therefore, to make comparisons between these figures and the doubtless more reliable data obtained by microbiological assay. The one serious discrepancy (on a percentage basis, if not in absolute amount) is in the case of histidine, where 1·2 % was found microbiologically and only 0·6 % by precipitation according to Kossel & Kutscher (1900–1) as silver salt, and re-precipitation as flavianate according to Vickery & Leavenworth (1927, 1928). The separation of histidine in the presence of much cystine appears to offer special difficulties. Argiris (1907–8) was unable to isolate any histidine from a similar type of hydrolysate (horse-hair). Vickery & Leavenworth (1929) found only 0·52 % of histidine in human hair. According to Dunn (1949) some laboratories fail to get satisfactory values in the microbiological estimation of arginine, histidine and lysine; no difficulties or sources of error were recognized in these determinations in our laboratory although the values for arginine and lysine were lower than by isolation. Significantly lower values (about 80 % of those estimated by isolation methods) were also obtained microbiologically for tyrosine. While the absolute magnitudes of the differences are small (at the worst less than 3 % of the keratin), these discrepancies are relatively large in percentage of the value found and would make a considerable difference in the molecular ratios if one were interested in calculating them. The fact that the values by microbiological

assay for the three amino-acids mentioned were smaller than those determined chemically might be explained either by inhibition of the assay organisms by the excessive amount of cystine in the hydrolysate or on the basis that some racemization had occurred during hydrolysis or, thirdly, that the fractions isolated by Beveridge & Lucas, in spite of good analytical values, were somewhat impure.

One is left with the impression that the higher values for arginine, lysine and tyrosine by the isolation procedures were probably due either to partial racemization giving low values in the microbiological assay, to extraneous amino-acids contaminating the fractions isolated, or to a combination of these effects. Unfortunately, suitable assay organisms are not known that respond equally well to both enantiomorphs of the amino-acids.

Table 1. *Analytical data obtained on human hair keratin*

(The values are reported on a moisture- and ash-free basis. (Letters in brackets indicate references.) A.T.C.C. = American Type Culture Collection, Washington D.C.)

Amino-acid	Test organism	%	Chemical method	%	Values reported by others
Alanine	1	2·8	Cu salt	(2·3+)	6·9 (*a*)
Arginine	2	7·8	Flavianate	8·9	9·4 (*b*)
Aspartic acid	3	3·9	Ba, Cu salts	3·5	—
Cystine	—	—	Colour*	18·0	21·2 (*c*); 16·5 (*d*)
Glutamic acid	4	13·6	Ba salt, HCl	10·6	8·0 (*a*)
Glycine	3	4·1	—	—	9·1 (*a*); 4·3 (*e*)
Histidine	2	1·2	Flavianate	0·6	0·6 (*e*)
Hydroxyproline	—	—	Reineckate	0	—
Isoleucine	3	4·8	Crystallization	(7+)	12·1 (*a*)
Leucine	4	6·4			
Lysine	3	1·9	Picrate	2·4	2·5 (*d*); 2·5 (*e*)
Methionine	2	0·7	Thiolactone†	0·8	—
Phenylalanine	3	2·4	—	—	2·6 (*e*)
Proline	3	4·3	Cu salt	4·3	—
Serine	5	10·6	HIO_4‡	8·0	—
Threonine	2	8·5	—	—	—
Tryptophan	—	—	Colour§	1	1·6 (*f*); 0·7 (*e*); 1·1 (*g*)
Tyrosine	3	2·2	Crystallization	2·5	4·3 (*g*); 3·3 (*a*); 3·0 (*e*)
Valine	4	5·5	Cu, Zn salts	(1·1+)	—
Melanin	—	—	—	1·6	—
Ammonia	—	—	—	1·42	—
N on inorg. ppts.	—	—	—	(1·13)	—

1, *Leuconostoc citrovorum* (A.T.C.C. 8081).
2, *Streptococcus faecalis* (A.T.C.C. 8043).
3, *Leuconostoc mesenteroides* P60 (A.T.C.C. 8042).
4, *Lactobacillus arabinosus* 17–5 (A.T.C.C. 8014).
5, *Lb. delbruckii* LD5 (A.T.C.C. 9595).

* Lugg (1933).
† Kassel & Brand (1938).
‡ Nicolet & Shinn (1941).
§ Spies & Chambers (1949).

REFERENCES

(*a*) Buchtala (1913).
(*b*) Vickery (1940).
(*c*) Wilson & Lewis (1927).
(*d*) Vickery & Leavenworth (1929).
(*e*) Block, Bolling, Brand & Schein (1939).
(*f*) Sammartino (1922).
(*g*) Klinke (1925).

A mixture of amino-acids of the composition shown in Table 2, which, according to the findings, approximates a hair hydrolysate, was prepared and analysed microbiologically for arginine, glutamic acid, histidine and threonine. The recoveries were 99, 101, 101 and 97 %, respectively, of the amounts used. Cystine does not seem to exert any inhibitory influence under these conditions. Some other as yet unrecognized compound might, of course, have an inhibiting effect.

Most of the determinations were repeated several times on different hydrolysates, in some cases with two or even three different organisms. A few typical sets of results will indicate the variations encountered: glycine 3·95, 3·98, 4·09, 4·11; histidine 1·14, 1·16, 1·23, 1·26, 1·30; leucine 6·30, 6·37, 6·42; valine 5·17, 5·40, 5·41, 5·48, 5·64, 5·81. Glutamic acid values as estimated by *Lactobacillus arabinosus* and by *Leuconostoc mesenteroides* P 60 were essentially the same: 13·14, 13·19, 13·21, 13·48, 13·55,

13·70, 13·82, 13·90, 13·94 with the first organism and 13·23, 13·64 and 13·99 with the second. *Streptococcus faecalis* gave somewhat lower values, 12·12, 12·30 and 12·97.

Although microbiological methods for glycine, alanine, proline and serine have not proven entirely satisfactory (Dunn, 1949), in this instance the value for proline agrees with that determined chemically. The value found for serine by chemical means (periodate) is 25% lower than that found using *Lactobacillus delbruckii* LD 5; it is difficult to decide which value may be nearer the truth.

The cystine value of 18·0% was adopted after a study of its rate of liberation. Hair was boiled with 9N-hydrochloric acid (5 ml./g.) and free cystine was estimated at intervals in the hydrolysate. A maximal value of just over 18% was obtained after 6 hr.: the value was 18·0% at 10 hr. and 16·4% after 24 hr. Merrill (1921) found maximal values for cystine in wool hydrolysates after boiling with acid for between 6 and 12 hr., and Rimington (1929) after 7 hr.: both noted that a slow decrease occurred on longer boiling. Sullivan & Hess (1937) found the cystine in casein to be completely liberated after 6–7 hr. boiling with 20% (w/v) hydrochloric acid. Vickery & Leavenworth (1929) give a value of 16·5% for the cystine content of human hair (determined after hydrolysis with 8N-hydrochloric acid for 8 hr.). If the higher figure of 18·0% is adopted, and a value of 0·7% for methionine is accepted, the sulphur content of the hair is practically accounted for (sulphur calculated from these data, 4·97; found, 5·00%).

By microbiological procedures about 81 g. of material in the hydrolysate from 100 g. of hair keratin have been identified. By isolation or chemical methods approximately 71 g. were characterized; the absence of one amino-acid (hydroxyproline) appears to have been established by chemical means. Melanin and ammonia increased the material accounted for by another 3·0 g. The total weight of material identified is about 104 g. (The mean molecular weight of the constituent amino-acids is, by calculation, about 152 so that 100 g. of keratin on hydrolysis should yield about 112 g. of split-products.) The failure to identify about 8 g. of material out of the expected 112 g. has been a disappointment. The amounts of the amino-acids shown in Table 2 represent 85·0% of the total nitrogen; ammonia and melanin account for another 7·7%. Thus, it has been possible to account for about 93% of the nitrogen of human hair keratin.

In the course of the chemical fractionation about 1·13 g. nitrogen, equal to 7% of the total nitrogen, was found adsorbed to inorganic precipitates in a form not removable by washing with water or dilute acid. This nitrogen, of as yet unrecognized nature, probably represents about 10 g. of amino-acids. Only if the bulk of the adsorbed nitrogen were in a form different from that of any of the 16 amino-acids determined microbiologically would one be justified in adding this 10% of the material weight or 7% of the total nitrogen to the 93% of each of these constituents already identified.

Table 2. *Amino-acid content of human hair keratin*

	In hair keratin (%)	N (g./100 g.)	N (as % of total)
Alanine	2·8	0·44	2·68
Arginine	8·9	2·87	17·50
Aspartic acid	3·9	0·41	2·50
Cystine	18·0	2·10	12·81
Glutamic acid	13·6	1·29	7·87
Glycine	4·1	0·77	4·68
Histidine	1·2	0·33	2·01
Hydroxyproline	0	0·0	0
Isoleucine	4·8	0·51	3·11
Leucine	6·4	0·68	4·15
Lysine	1·9	0·36	2·22
Methionine	0·7	0·07	0·43
Phenylalanine	2·4	0·20	1·22
Proline	4·3	0·52	3·18
Serine	10·6	1·41	8·62
Threonine	8·5	1·00	6·10
Tryptophan	1	0·14	0·85
Tyrosine	2·2	0·17	1·04
Valine	5·5	0·66	4·02
Melanin	1·6	0·10	0·61
Ammonia	1·42	1·17	7·13
Unaccounted for	(8·2)	1·2	7·3
Total	112	16·4	100

Because different regions of the hair shaft may be distinguished histologically, it would appear reasonable to assume that different proteins may be concerned in its make-up. Consequently it would seem foolish to consider that the keratin material analysed represents a single compound. When the percentages found were divided by the respective molecular weights to get some idea of the relative frequency of occurrence of the amino-acids, a series of numbers not too far removed from simple ratios were obtained. Even if no assumption is involved as to the homogeneity of the material analysed, it is interesting to note that the frequency of occurrence of the amino-acids, on a molecular basis, appears to be roughly as follows: serine 32, glutamic acid 30, cystine 25, threonine 24, glycine 18, arginine 16, leucine 16, valine 16, isoleucine 12, proline 12, alanine 10, aspartic acid 10, phenylalanine 5,

lysine 4, tyrosine 4, histidine 2, methionine 2 and tryptophan 2.

SUMMARY

1. Values are given for nineteen of the common amino-acids in human hair keratin.

2. These account for about 90 % of the expected weight and 86 % of the nitrogen of the keratin; melanin plus ammonia account for another 3 % of the weight and 7·7 % of the nitrogen.

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REFERENCES

Argiris, A. (1907–8). *Hoppe-Seyl. Z.* **54**, 86.
Beveridge, J. M. R. & Lucas, C. C. (1944*a*). *Biochem. J.* **38**, 88.
Beveridge, J. M. R. & Lucas, C. C. (1944*b*). *Biochem. J.* **38**, 95.
Beveridge, J. M. R. & Lucas, C. C. (1944*c*). *Biochem. J.* **38**, 411.
Block, R. J., Bolling, D., Brand, F. C. & Schein, A. (1939). *J. biol. Chem.* **128**, 181.
Block, R. J. & Vickery, H. B. (1931). *J. biol. Chem.* **93**, 113.
Buchtala, H. (1913). *Hoppe-Seyl. Z.* **85**, 246.
Dunn, M. S. (1949). *Physiol. Rev.* **29**, 219.
Henderson, L. M. & Snell, E. E. (1948). *J. biol. Chem.* **172**, 15.
Kassel, B. & Brand, E. (1938). *J. biol. Chem.* **125**, 145.
Klinke, K. (1925). *Biochem. Z.* **160**, 28.
Kossel, A. & Kutscher, F. (1900–1). *Hoppe-Seyl. Z.* **31**, 165.
Lucas, C. C. & Beveridge, J. M. R. (1940). *Biochem. J.* **34**, 1356.
Lugg, J. W. H. (1933). *Biochem. J.* **27**, 668.
Merrill, A. T. (1921). *J. Amer. chem. Soc.* **43**, 2688.
Nicolet, B. H. & Shinn, L. A. (1941). *J. biol. Chem.* **139**, 687.
Rimington, C. (1929). *Biochem. J.* **23**, 41.
Sauberlich, H. E. & Baumann, C. A. (1949). *J. biol. Chem.* **177**, 545.
Sammartino, U. (1922). *Biochem. Z.* **133**, 476.
Spies, J. R. & Chambers, D. C. (1949). *Anal. Chem.* **21**, 1249.
Stokes, J. L. & Gunness, M. (1945). *J. biol. Chem.* **157**, 651.
Sullivan, M. X. (1926). *U.S. Public Health Reports*, **41**, 1030.
Sullivan, M. X. & Hess, W. C. (1937). *J. biol. Chem.* **117**, 423.
Vickery, H. B. (1940). *J. biol. Chem.* **132**, 325.
Vickery, H. B. & Leavenworth, C. S. (1927). *J. biol. Chem.* **72**, 403.
Vickery, H. B. & Leavenworth, C. S. (1928). *J. biol. Chem.* **76**, 707.
Vickery, H. B. & Leavenworth, C. S. (1929). *J. biol. Chem.* **83**, 523.
Wilson, R. H. & Lewis, H. B. (1927). *J. biol. Chem.* **73**, 543.

Further Isotopic Studies on Haemoglobin Formation in the Rat and Rabbit

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Recent work done *in vivo* with rabbits (Muir & Neuberger, 1950) and *in vitro* with nucleated red cells of birds (Radin, Rittenberg & Shemin, 1950) has shown that the α-carbon atoms of glycine molecules provide eight carbon atoms for the porphyrin moiety of haemoglobin. Earlier investigations (Wittenberg & Shemin, 1949; Muir & Neuberger, 1949) had produced evidence that all four nitrogen atoms of porphyrin are also specifically derived from glycine. The present paper is concerned in the first place with a comparison of the isotope contents of the porphyrin and glycyl residue in rabbit haemoglobin at different time intervals after administration of methylene-labelled glycine. If glycine is indeed a specific precursor of porphyrin, it would be expected that the ratio of 'molar' radioactivities of porphyrin and globin glycine should approach or be equal to the value of eight, provided that the rate of blood formation is constant and that globin and porphyrin are made in the same cell or cell compartment, during the experiment, i.e. utilize the same glycine 'pool'. It is of course possible that the newly formed porphyrin or globin are diluted to different extents by unlabelled preformed material or that the average times required for the incorporation of the α-carbon atoms of glycine into the porphyrin and globin moieties of haemoglobin differ sinificantly. In

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either of these cases the shapes of the activity-time curves of these two components will not be identical initially, but the ratio of the molar activities should approach eight, as the curves flatten out, provided the above conditions apply. This investigation was then extended to the rat whose haemoglobin, unlike that of the rabbit, can readily be obtained in a crystalline form (Warburg & Reid, 1931). Preliminary experiments had shown that valyl residues provide the terminal amino groups of the peptide chains of rat haemoglobin, as in the haemoglobins of many other species (Porter & Sanger, 1948). We have therefore injected radioactive glycine and valine in constant proportion to groups of rats and measured the activities of porphyrin, the terminal valine residues, the non-terminal valine residues and the glycine residues at different times after administration of the labelled compound. It was hoped that the data thus obtained would be susceptible to a less ambiguous and more quantitative interpretation than is generally the case with isotope results on protein metabolism, for the following reasons. Haemoglobin isolated from adult mammals, unlike most other intracellular proteins, can be considered to be reasonably homogeneous, as measured by known physical and chemical criteria. Moreover, the haemoglobin of the normal, mature erythrocyte of the mammal is metabolically either completely or almost completely inert and we are justified in interpreting the activity-time curves of haemoglobin obtained for the first weeks after administration of labelled precursors entirely in terms of protein synthesis and in neglecting degradation.

In these experiments protein fractions were obtained which were almost free from haemoglobin and which were derived largely from the red cell membranes. These heterogeneous fractions which were designated 'stromatin', were also hydrolysed and the radioactivities of the valine and the glycine determined. In these and the haemoglobin experiments the rather small amounts of amino-acids available were isolated in the form of 2:4-dinitrophenyl (DNP) derivatives by a method described in detail elsewhere (Perrone, 1951).

EXPERIMENTAL

Treatment of animals and administration of labelled compounds

Rabbit experiments. In the first rabbit experiment a female Hollingsworth half-lopped rabbit (body weight 2·75 kg.) was used. The animal received 57·4 μc. of [α-^{14}C]-glycine having a specific activity of 1 μc./mg. glycine and 100 μc. of [*carboxy*-^{14}C] acetate (8·3 mg. anhydrous sodium acetate) intravenously. At stated intervals blood samples (10 ml. each) were removed from the marginal ear vein. In the second rabbit experiment a Dutch female rabbit (3·7 kg. body weight) was given, by vein, an isotonic solution containing 20 mg. of [α-^{14}C]glycine with a total activity of approx. 300 μc. The rabbit was killed 8 hr. after the admistration of the glycine. This animal was primarily used for a study of milk protein formation by Dr Campbell and Dr Work, to whom we are grateful for supplying the blood.

A third rabbit (3·3 kg. body weight female half-lopped Hollingsworth) was given a low protein diet for 48 hr. and then fasted for 24 hr. It was then given by mouth 7 μc. of [α-^{14}C]lysine monohydrochloride (460 mg.) admixed with a small amount of pelleted diet. The lysine which was mainly in the L-form, but contained approx. 15% of the D-isomer and which had been synthesized in this laboratory (Arnstein, Hunter, Muir & Neuberger, 1952). Blood samples (10 ml. each) were taken from the marginal ear vein at stated intervals. We are grateful to Dr A. S. McFarlane for supplying us with the washed corpuscles for this experiment.

Rat experiments. Male and female rats of the Institute albino strain (200–270 g. body weight) were used. Each rat was injected intraperitoneally with an amount of a solution containing 5 μc. of [α-^{14}C]glycine and 3·5 μc. of [*gem*-methyl-^{14}C]DL-valine/100 g. body weight. The glycine had a specific activity of 1 μc./mg. and the valine a specific activity of 1 μc./0·7 mg. Pairs of rats were killed by ether at various stated intervals of time and the blood removed by heart puncture. The labelled compounds excepting lysine were obtained from the Radiochemical Centre, Amersham.

Isolation of substances

Haemoglobin. In the first rabbit experiment the washed corpuscles were laked with distilled water and the haemoglobin precipitated by pouring the haemolysate into 10 vol. of acetone. The precipitate was further extracted successively with boiling acetone and $CHCl_3$. The extracts were used for the preparation of cholesterol (see Muir, Perrone & Popják, 1951). In the second and third rabbit experiments the washed red cells, after removal of the 'buffy coat' containing the leucocytes, were lysed by addition of distilled water and the 'stromatin' precipitated by passage of CO_2 into the mixture. The solution was centrifuged and the precipitate discarded. Rabbit haemoglobin could not be crystallized, but the preparations obtained by the second method appeared to be uniform on electrophoresis.

In the rat experiments the washed red cells, after removal of the white cell layer, were lysed by addition of 4 vol. of water saturated with CO_2. The precipitate containing the 'stromatin' was removed on the centrifuge. The haemoglobin was crystallized by addition of acetone (0·3 vol.) and leaving the mixture at 0° for 18 hr. The haemoglobin was recrystallized by dissolving the crystals in CO_2-saturated water followed by addition of acetone (0·3 vol.) and cooling to 0°. The 'stromatin' was washed repeatedly with CO_2-saturated water, until no more haemoglobin could be detected in the supernatant fluid.

Porphyrin and globin. Protoporphyrin methyl ester was obtained from the rabbit haemoglobin by the method of Grinstein (1947); the residue insoluble in acidic methanol was used for the preparation of amino-acid derivatives. In the rat experiments the globin was prepared by the method of Anson & Mirsky (1930). The supernatant solution from the acetone-HCl treatment was evaporated to dryness and the protoporphyrin methyl ester obtained from the residue according to Grinstein (1947). In the one experiment in which a degradation was carried out, the protoporphyrin

ester was reduced to mesoporphyrin ester and the latter oxidized with CrO_3 as described by Muir & Neuberger (1950).

Amino-acids. The proteins were hydrolysed and DNP-glycine prepared as described by Perrone (1951). In the rat experiments the globin was treated with 1:2:4 fluorodinitrobenzene (FDNB) (Sanger, 1945; Porter & Sanger, 1948) and the DNP-globin was hydrolysed. Extraction with ether and chromatographic separation (Perrone, 1951) gave crystalline DNP-L-valine derived from 'end-group' valine. The remaining free amino-acids were again treated with FDNB and the crystalline DNP-glycine and DNP-L-valine derived from non-terminal valine were obtained from this fraction. Lysine was obtained from the phosphotungstic acid-insoluble fraction of the globin hydrolysate by precipitation as copper-benzylidene complex (Turba, 1948); this was decomposed with H_2S and the lysine isolated as dihydrochloride after removal of the benzaldehyde with ether.

Isotope determinations

Radioactivities were measured as described (Muir & Neuberger, 1950), using an experimentally determined correction curve, if the samples were smaller than 20 mg./sq.cm. The results are given as counts/min. for samples of 'Infinite' thickness and 1 sq.cm. area. In an earlier paper (Muir & Neuberger, 1950) activities were expressed in an identical manner but erroneously defined as counts/min./mg. sample. In order to facilitate comparisons between different molecular species these values are multiplied by the molecular weight and divided by 1000. Such corrected figures are termed 'molecular activities'.

RESULTS

Comparison between the radioactivities of the porphyrin and the glycine residue in globin

In the rabbit experiment (Table 1) appreciable radioactivity was found both in the porphyrin and glycine of the globin as early as 6 hr. after the administration of labelled glycine. The maximum activity was reached after approximately 3 days; after the seventh day activity decreased and had fallen to about 40% of its highest value by the fifty-second day of the experiment, suggesting an average half-life span for the rabbit erythrocyte of about 40 days. This figure is lower than the values obtained in earlier experiments with ^{15}N (Neuberger & Niven, 1951). The difference may be explained as follows: the blood samples taken in the present investigation were rather large (10 ml.) and by the twenty-second day approximately 30% of the initial labelled haemoglobin had been removed and this was presumably replaced by protein of negligible isotope content. Moreover, the particular rabbit used increased its body weight during the experiment and probably also its blood volume by about 12%. These two factors thus account for the apparently short life-span, but they will not affect the ratio of activity between porphyrin and globin glycine in which we were primarily interested.

Table 1. *Radioactivities of samples of protoporphyrin methyl ester and of DNP-glycine obtained at various times from haemoglobin specimens of a rabbit which had received* 57·4 *μc. of* [α-^{14}C]*glycine*

(The values for molar radioactivity were obtained by multiplying the observed counts/min. by the mol.wt. and dividing by 1000.)

	Times of collection after administration of labelled compound (days)					
Porphyrin ester:	0·25	1·0	3·0	7·0	22·0	52·0
Counts/min.	49·9	224·9	391·6*	476·6	386·8	178·0
Molar radioactivity	29·45	132·6	240·5*	281·2	228·5	105·0
DNP-glycine from globin:						
Counts/min.	23·4	80·46	145·2	127·4	112·6	54·3
Molar radioactivity	5·64	19·64	35·0	30·7	21·7	13·1

* This sample amounted to 11·0 mg. only; correction to infinite thickness is given for molar radioactivity only, whilst counts/min. represents the value observed.

The relatively small amount of carboxyl-labelled acetate which was given to the rabbit, together with the labelled glycine, will produce a very slight activity in the carboxyl groups of protoporphyrin. However, the dilution factor for acetate is very large compared with that of glycine and we have therefore assumed in our calculations that the whole activity of the porphyrin is derived from the α-carbon atom of glycine.

In the rat experiment (Table 2) two animals were used for each time interval. Although the animals were kept under identical conditions and doses of radioactive amino-acids were exactly proportional to body weight, individual differences were not entirely eliminated. Such differences, which will affect the absolute values, should not influence to the same extent the ratios of radioactivities of different components of haemoglobin and it is only the ratios which are being considered.

Table 3 gives the ratios of molar activities of porphyrin and globin glycine from both rat and rabbit experiments for different time intervals. If we consider in the first place only those values which were obtained 24 hr. or more after administration of labelled glycine, we find that the mean molar activity of porphyrin is about 7·7 times that of the glycine isolated from the same haemoglobin sample.

This mean figure is reasonably close to the value of 8 which is expected on the basis of earlier work based on degradation of porphyrin (Muir & Neuberger, 1950; Wittenberg & Shemin, 1950). However, in these experiments the assumption was made that the radioactivity or the ^{15}N content of the 1950) and the α-carbon atom of the glycine residue in globin have identical activities within ±10 or 15%.

In haemoglobin samples taken 6 or 12 hr. after administration of radioactive glycine, the activity ratio was significantly less than 8, the values varying

Table 2. *Radioactivities of samples of protoporphyrin methyl ester and of DNP-glycine obtained from crystalline haemoglobin specimens of rats which had received 5 μc. [α-^{14}C] glycine and [gem-methyl-^{14}C] DL-valine/100 g. body weight*

(Two rats were used for each experiment.)

	Time of collection after administration of labelled compounds (days)					
Porphyrin ester:	0·5	1·0	3·0	7·0	14	21
Counts/min.	162·5	N.d.	419·9	297·8	375·6	326·5
Molar radioactivity	95·9	N.d.	247·7	175·8	221·6	192·3
DNP-glycine from globin:						
Counts/min.	73·5	70·5	125·0	104·8	136·0	95·71
Molar radioactivity	17·7	17·0	30·1	25·3	32·8	23·1

N.d., not determined.

Table 3. *Ratio of molar radioactivities in rats and rabbits of protoporphyrin and glycine obtained from haemoglobin samples obtained at different times after administration of labelled glycine*

(The molar activities are given in Tables 1 and 2.)

Rabbits		Rats	
Time after administration of [α-^{14}C]glycine (days)	Molar activity of porphyrin/ molar activity of globin glycine	Time after administration of [α-^{14}C]glycine (days)	Molar activity of porphyrin/ molar activity of globin glycine
0·25	5·2	0·5	5·4
0·25	5·6	3·0	8·2
1·0	6·8	7·0	7·0
3·0	6·9	14·0	6·8
7·0	9·1	21·0	8·3
22·0	8·4	—	—
52·0	8·0	—	—

Table 4. *Comparison of radioactivities of products of oxidation of mesoporphyrin**

(The distribution of radioactivities was obtained by multiplying the molar activities shown in the second line by 2 in the case of haematinic acid and methylethylmaleic acid, and by 6 in the case of barium carbonate.)

	Mesoporphyrin methyl ester	Haematinic acid	Methylethylmaleic acid imide	Barium carbonate
Counts/min.	295·2±4·3	108·7±1·2	145·5±2·4	71·4±0·7
$\frac{\text{Counts/min.} \times \text{mol.wt.}}{1000}$	175·2±2·6	19·9±0·2	19·94±0·3	14·09±0·13
Percentage distribution of radioactivity	(100)	22·72	22·77	48·27

* Mesoporphyrin isolated from second rabbit diluted with inactive mesoporphyrin.

maleimide was derived to an equal extent from rings *A* and *B* of protoporphyrin and the activity of haematinic acid from rings *C* and *D*. The unlikely possibility that only two rings, one of each type, and two methene carbon atoms were labelled was not completely excluded. The present results clearly justify the assumption made in the earlier interpretation and suggest that all four methene carbon atoms, all carbon atoms in the 2-positions of the pyrrolic rings (notation of Wittenberg & Shemin, between 5·2 and 5·6. These values are well outside the limits of variation found with the later samples. The possibility was considered that this was due to the complexity, and hence possible slowness of the reactions leading from glycine to either the methene groups or to the formation of the pyrrole rings. If one of these reactions were slow in relation to the other, a degradation of a porphyrin specimen obtained a few hours after administration of glycine, should reveal considerable differences in the relative

activities of the degradation products. Such an experiment (Table 4) carried out with a haemoglobin sample obtained 6 hr. after administration of labelled glycine gave results which were more or less identical with those obtained with a haemoglobin specimen obtained several days after administration of labelled glycine (Muir & Neuberger, 1950). In fact, agreement with theory was better than in the earlier experiments. It can therefore be concluded that the rates of conversion of the α-carbon atom of glycine to the methene groups and its incorporation into the pyrrolic rings must be of similar magnitude.

The incorporation of labelled glycine and valine into stromatin

The quantities of stromatin available were rather small and thus only about 2–3 mg. of DNP-glycine and DNP-valine were obtained in most cases; however, the samples were chromatographically pure and had correct melting points. The DNP-derivatives were diluted as described in Table 6 and the accuracy of the activity measurements is somewhat lower than with the globin samples. Comparison between the activity-time curves of the two amino-acids in globin and stromatin shows that the activities of both the DNP-glycine and the DNP-valine obtained from the 12 hr. stromatin specimens were very low compared with the corresponding values of globin (Tables 6 and 7). In the case of 3-day specimens the activities of the stromatin samples are

Table 5. *Radioactivities of DNP-valine isolated from terminal and non-terminal positions of rat globin samples obtained at various intervals after administration of labelled valine and glycine*

(Values for molar activity of globin glycine were taken from Table 2.)

Time after administration of labelled amino-acids (days)	Radioactivity of terminal valine		Radioactivity of non-terminal valine		Ratio of molar activity of terminal over non-terminal valine	Molar activity of glycine from globin	Ratio of molar activity of glycine over non-terminal valine
	(Counts/min.)	Molar activity	(Counts/min.)	Molar activity			
0·5	S.L.	S.L.	29·15	8·3	S.L.	17·7	2·13
1·0	33·1	9·36	33·1	9·36	1·00	17·0	1·82
3·0	74·2	21·1	76·1	21·5	0·98	30·15	1·40
7·0	58·1	16·5	59·5	16·8	0·98	25·2	1·50
14·0	102·7	29·2	102·0	29·1	1·00	32·8	1·13

S.L., sample lost.

The rate of incorporation of valine into globin

Table 5 shows that the rates of incorporation of radioactive valine in terminal and non-terminal positions of the peptide chains of the globin molecule are identical. The very small differences found were well within the limits of error of counting. A comparison between the activities of glycine and of valine obtained from the same globin samples indicates that the ratio of the two activities decreases somewhat with time. A very similar change was found in a comparison of a change of ratio of the activities with time in the case of stromatin (Table 7).

The incorporation of L-[α-^{14}C]*lysine into globin*

This experiment was carried out with material of relatively low specific activity; moreover, the total activity injected was small. The counting errors in the early samples were therefore large. The following values (counts/min.) on disks of 2 sq.cm. were obtained on samples of L-lysine-dihydrochloride (infinite thickness): 2 hr., 0; 6 hr., 3·3; 10 hr., 4·5; 14 hr., 15·2; 19 hr., 31·5; 24 hr., 38·4; and 29·5 hr., 52·6. (The times in hours indicate the intervals between administration of lysine and collection of samples.) The protoporphyrin methyl ester isolated from the haemoglobin sample which had been collected 29·5 hr. after administration of labelled lysine was completely inactive.

Table 6. *Radioactivities of DNP-glycine isolated from stromatin samples obtained at various intervals after simultaneous administration of labelled glycine and valine*

(The amounts of DNP-glycine obtained from stromatin were generally too small to be counted directly under conditions of 'infinite thickness'. The specimens after recrystallization were therefore dissolved in ether; a known amount of unlabelled DNP-glycine was added to a solution of the labelled DNP-glycine which had also been carefully weighed. The mixture was then evaporated to dryness and the crystalline residue counted. The dilution was generally threefold. The molar activities given have been corrected for this dilution effect. Values for molar activity of globin glycine were taken from Table 3.)

Time after administration of labelled amino-acids (days)	Molar activity of stromatin glycine	Molar activity of globin glycine	Ratio of molar activity of globin glycine over that of stromatin glycine
0·5	6·15	17·7	2·9
3·0	40·1	30·15	0·75
7·0	23·2	25·2	1·09
14·0	25·0	32·8	1·31

higher, to fall again relative to globin in the 1-week and 2-week samples. It is interesting that the ratios of the molar activities of valine and of glycine in stromatin and globin respectively were so similar for identical time intervals.

DISCUSSION

The interrelationship between the formation of porphyrin and that of globin

It is generally agreed that both components of the haemoglobin molecule, i.e. haem and globin are synthesized in the maturing erythrocyte. From microscopic observation it had been known for some time that the appearance of pigment occurs relatively late in the development of the red cell, and recently Thorell (1947) has shown by the use of spectroscopic methods that formation of the bulk of the protein precedes the synthesis of porphyrin. The methods used by Thorell, which depend on selective ultraviolet absorption, did not enable him to demonstrate that this protein is in fact globin or chemically related to globin and the sequence of the two processes, formation of globin and of porphyrin, cannot be deduced from these observations. Experiments done *in vitro* with homogenates of spleen and bone marrow of rabbits and rats (Richmond, Altman & Salomon, 1951) and *in vivo*, with rats (Salomon, Altman, Casarett & Noonan, 1949), have shown that exposure of the living animal to X-rays affects the synthesis of haem and of globin to a different extent. Thus it appears that the two processes are largely independent of one another, although they occur in the same cell and lead to a common end product, haemoglobin.

The relative rates of incorporation of radioactive carbon derived from α-labelled glycine into the porphyrin and globin, respectively, have already been measured in the rat by Altman, Casarett, Masters, Noonan & Salomon (1948) who followed the isotope contents of the two components over a considerable period of time. However, their results are not easy to interpret quantitatively, since only the activity of the globin and not that of the glycine was determined. The glycine originally administered will give rise to activity in other amino-acids and the fraction of the radioactivity of the whole protein not due to glycine is likely to increase with time. In the present experiments a crystalline glycine derivative was isolated and its activity compared with that of porphyrin. It was found both in the rat and rabbit (at least with samples obtained 24 hr. or more after administration of the labelled glycine), that the ratios of the molar activities of glycyl residue in globin and of the porphyrin were within ± 15 % of a constant value. This result is to be expected on the basis of the concept of the steady state, provided that the two components are formed from the same glycine 'pool'. The postulate of the steady state requires that both the amounts of the substances under consideration and their rates of synthesis from the common precursor should be constant during the period of the experiment. As already pointed out, the fact that this ratio of molar activities is approximately 8 agrees well with current theories which assume that eight carbon atoms of protoporphyrin are exclusively derived from the α-carbon atom of glycine.

With haemoglobin samples obtained 6 or 12 hr. after administration of labelled glycine, the molar activity of the porphyrin was only about 5·0 to 5·5 times higher than that of the DNP-glycine. This high isotope content of the globin glycine relative to that of the porphyrin found with the early samples could be explained by assuming that the newly formed labelled globin combines with preformed unlabelled haem. However, this deduction, which implies that porphyrin formation precedes globin synthesis, is only valid if the times required for the incorporation of the labelled α-carbon atom of glycine into the various positions of the porphyrin moiety and of the glycyl residue of the globin are the same. It is possible that since the reactions which lead from glycine to protoporphyrin or haematin are more complex than the incorporation of the glycine residue into globin, the former reactions will take longer. Hence the first cells which appear in the circulation after administration of glycine, will contain a higher proportion of isotope in the globin than in the haem. The time relationships in relative activities will also be affected by the size of the 'pools' of the glycine, the protoporphyrin and the various intermediates, and in the absence of any knowledge on these points it would be hazardous to draw conclusions from the differences observed. In any case the relatively high labelling found in these early samples suggests that both components are synthesized at a relatively late stage in the development of the red cell.

The rate of incorporation of labelled valine and glycine into globin

The rate at which labelled amino-acid residues appear in the haemoglobin of circulating red cells is determined by a number of factors. The time required for the synthesis of the protein from the amino-acids is probably quite short, but the interval between the synthesis of haemoglobin in the erythroblast and the release of the reticulocyte from the bone marrow may vary between species and may not be constant for different red cells of the same animal. Moreover, haemoglobin formation occurs with varying intensity during different morphological stages in the development of the cell and thus the activity-time curves of the amino-acids obtained from haemoglobin of circulating erythrocytes do not

accurately reflect the rate of incorporation of the labelled amino-acids into globin. But apart from these factors which are concerned with the physiology of the cell, we must also consider the changes in activity of the free intracellular amino-acids which are presumably the direct precursors of the protein. The isotope content of the free amino-acid will rise very quickly, on parenteral administration, to a maximum, at least in actively metabolizing and highly vascularized tissue such as bone-marrow. This will be followed by a sharp decrease in isotope content owing to the occurrence of fast metabolic reactions and distribution of the amino-acid over tissues with relatively low permeability. During the third period the isotope content will probably decrease relatively slowly to an asymptotic value of zero (for discussion, see Arnstein & Neuberger, 1951*a*). Thus some of the factors which will determine the change of isotope content of one particular amino-acid in a given cell are the following: permeability characteristics of the tissues for the compound under consideration, the rate of conversion to other substances, the rates of protein formation and of synthesis of the amino-acid from unlabelled precursors. It can be expected that these factors will operate in a quantitatively different manner with different amino-acids. Valine, for example, cannot be synthesized by the animal and dilution will therefore be largely due to dietary intake and liberation of the amino-acid by hydrolysis of proteins. Glycine, on the other hand, is synthesized at the rate of about 2 m-moles/100 g. of rat (Arnstein & Neuberger, 1951*b*) and it can be predicted that the isotope content of the free amino-acid will decrease more rapidly with time than that of valine. It is expected, therefore, that the specific activity of the valine incorporated into protein per unit time should decrease more slowly than that of glycine. This prediction is borne out by the data presented in Tables 5 and 7 which show that the ratios of the activities of DNP-glycine to DNP-valine tend to decrease with time both in globin and stromatin.

The dose of radioactive glycine injected was 5 μc./100 g. as compared with 3·5 μc./100 g. for DL-valine. A difficulty in interpreting the observed relative dilution factors arises from the fact that the injected valine was racemic and the exact proportion of the D-isomer which is inverted and becomes available for protein synthesis is unknown. An inversion in the case of leucine was demonstrated by Ratner, Schoenheimer & Rittenberg (1940), and the same probably applies to valine. However, the nutritional experiments of Rose (1938) suggest that such an inversion, if it takes place, is inconsiderable in the rat. Allowing for the difference in dosage and assuming that D-valine is not inverted to a significant extent, the data of Tables 5 and 7 suggest that radioactive glycine is diluted between two or three times as much as valine, the ratio changing with time for reasons discussed above.

Table 7. *Radioactivities of DNP-valine isolated from rat stromatin samples which had been collected at various intervals after administration of labelled valine and glycine*

(Dilution of samples was done as described in Table 6. The molar activities are corrected for dilution. Values for molar activities of globin valine and stromatin glycine are given in Tables 5 and 6 respectively.)

Time after administration of labelled amino-acids (days)	Molar activity of DNP-valine from stromatin	Molar activity of DNP-valine from globin	Ratio of molar activity of valine from globin over that of stromatin	Ratio of molar activity of stromatin glycine over stromatin valine
0·5	3·7	8·3	2·2	1·66
3·0	28·8	21·5	0·73	1·39
7·0	18·8	16·8	0·88	1·23
14·0	22·3	29·1	1·31	1·08

It was found that the ratio of the activities of the terminal valine to that of the non-terminal valine was unity and this suggests that both types of residue are incorporated into globin at approximately the same time. We do not know how far the two types of valine are separated from one another in the peptide chains of the globin molecule, but unless all or most of the valine groups are situated near the end of the chain the results suggest that the average time interval between the synthesis of a peptide involving a valine residue in terminal position and that of a peptide involving a valine residue in a non-terminal position is very short compared with the rate of decrease of isotope content in the free valine of the cell. These findings are compatible with either a mechanism of globin synthesis involving simultaneous condensation of amino-acids or, more probably, a process of rapid successive condensations involving intermediates of relatively short life. Anfinsen & Steinberg (1951) have recently found a considerable difference in isotope content between aspartic acid residues present in a peptide which is liberated from egg albumin during the conversion of the latter to plakalbumin and the aspartic acid residues present in the rest of the albumin molecule. It is possible that this system represents a special case or that different

mechanisms of synthesis operate with different proteins. Further work with homogeneous proteins is obviously required before any more general conclusions can be drawn.

The findings reported here with respect to the isotope contents of the protein and porphyrin after administration of labelled lysine indicate definitely that lysine is not a specific precursor of any part of the porphyrin molecule. This conclusion is in complete agreement with the theory based on degradation experiments that glycine and glutamic acid, or their derivatives, are the only specific precursors of protoporphyrin (see Neuberger, Muir & Gray, 1950; Shemin & Wittenberg, 1951). The finding of Altman, Miller & Richmond (1950) that labelled lysine gives rise to labelling in protoporphyrin may be explained, as already suggested by Altman *et al.* (1950), by conversion to glutamic acid which has been shown to occur (Miller & Bale, 1949).

The metabolic behaviour of stromatin

The experiments with stromatin were done in order to find out whether the proteins of the cell membrane are as inert metabolically as haemoglobin. The results obtained are not conclusive. The protein mixture termed stromatin is obviously very heterogeneous and may not comprise all the proteins of the membrane and may also contain proteins derived from the interior of the cell. The low isotope contents in the first glycine (Table 6) and valine sample (Table 7) compared with the corresponding values for globin indicate that the bulk of the proteins of this fraction are formed earlier than the haemoglobin. The values found in the other experiments are not greatly different from those observed for globin and this suggests that the bulk of the proteins comprising this fraction are, once formed, metabolically as inert as haemoglobin. If stromatin contained a large fraction with a turnover rate similar, for example, to that of plasma proteins, we should have found initial isotope contents very much higher than those of globin. The apparent decrease in activity relative to that of globin which was observed with both glycine and valine, may indicate that stromatin contains a fraction which is in the dynamic state even in the mature cell. However, the present data are not sufficient for definite conclusions to be drawn.

Most of the results given here have already been presented by one of us at an isotope conference (Neuberger, 1951). The present data, however, are more extensive and the interpretations have been slightly modified.

SUMMARY

1. The incorporation of [α-^{14}C]glycine into the protoporphyrin and globin moieties of the haemoglobin of rats and of a rabbit has been determined at various intervals of time after administration of glycine. Labelled valine was also administered to the rats.

2. After intervals of time greater than 24 hr. the molecular radioactivity of protoporphyrin was approximately 8 times greater than that of the glycine isolated from the same sample of haemoglobin, in agreement with current theories of haem synthesis. After shorter time intervals smaller values were obtained. Possible explanations are discussed.

3. Valine was also isolated from the 'end-groups' and remainder of the rat haemoglobin molecule. The radioactivities of both fractions were identical at all intervals of time. The implications of this finding are discussed.

4. The relative radioactivity of glycine to that of valine isolated from haemoglobin and from the heterogeneous protein 'stromatin' decreased as time went on. This is attributed to increasing dilution of radioactive glycine by endogenous synthesis.

5. The isotope contents of glycine and valine isolated from 'stromatin' and haemoglobin were compared. It is concluded that stroma proteins are formed earlier and are for the most part as metabolically inert as haemoglobin.

REFERENCES

Altman, K. I., Casarett, G. W., Masters, R. E., Noonan, T. R. & Salomon, K. (1948). *J. biol. Chem.* **176**, 319.
Altman, K. I., Miller, L. L. & Richmond, J. E. (1950). *Arch. Biochem.* **29**, 447.
Anfinsen, C. B. & Steinberg, D. (1951). *J. biol. Chem.* **189**, 739.
Anson, M. L. & Mirsky, A. E. (1930). *J. gen. Physiol.* **13**, 121.
Arnstein H. R. V., Hunter, G. D., Muir, H. M. & Neuberger, A. (1952). *J. chem. Soc.* p. 1329.
Arnstein, H. R. V. & Neuberger, A. (1951*a*). *Biochem. J.* **50**, 154.
Arnstein, H. R. V. & Neuberger, A. (1951*b*). *Biochem. J.* **48**, ii.
Grinstein, M. (1947). *J. biol. Chem.* **167**, 515.
Miller, L. L. & Bale, W. F. (1949). *Fed. Proc.* **8**, 230.
Muir, H. M. & Neuberger, A. (1949). *Biochem. J.* **45**, 163.
Muir, H. M. & Neuberger, A. (1950). *Biochem. J.* **47**, 97.
Muir, H. M., Perrone, J. C. & Popják, G. (1951). *Biochem. J.* **48**, iv.
Neuberger, A. (1951). *Ciba Foundation Conference on Isotopes in Biochemistry*, part II, p. 68. London: J. and A. Churchill.
Neuberger, A., Muir, H. M. & Gray, C. H. (1950). *Nature, Lond.*, **165**, 948.
Neuberger, A. & Niven, J. S. F. (1951). *J. Physiol.* **112**, 292.
Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.

Radin, N. S., Rittenberg, D. & Shemin, D. (1950). *J. biol. Chem.* **184**, 745.
Ratner, S., Schoenheimer, R. & Rittenberg, D. (1940). *J. biol. Chem.* **134**, 653.
Richmond, J. E., Altman, K. I. & Salomon, K. (1951). *J. biol. Chem.* **190**, 817.
Rose, W. C. (1938). *Physiol. Rev.* **18**, 109.
Salomon, K., Altman, K. I., Casarett, G. W. & Noonan, T. R. (1949). *Fed. Proc.* **8**, 247.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Shemin, D. & Wittenberg, J. (1951). *J. biol. Chem.* **192**, 315.
Thorell, B. (1947). *Acta med. scand.* **129** (Suppl.), 200.
Turba, F. (1948). *Hoppe-Seyl. Z.* **283**, 19.
Warburg, O. & Reid, A. (1931). *Biochem. Z.* **242**, 149.
Wittenberg, J. & Shemin, D. (1949). *J. biol. Chem.* **178**, 47.
Wittenberg, J. & Shemin, D. (1950). *J. biol. Chem.* **185**, 103.

Lignin in Young Plants

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In a previous communication (Bondi & Meyer, 1948) we showed that lignins extracted by alkali ('alkali lignin') from annual plants yield a number of aromatic degradation products similar to those obtained from wood lignins. The preparation of lignin from faeces collected from sheep fed one plant exclusively was used to investigate the changes occurring in lignin during digestion in the ruminant body. It was found that the lignin molecule is not attacked to any appreciable degree during its passage through the animal body. It was found, however, that the alkali-extractable lignins differed considerably both in yield and composition from those obtained by the usual methods using acid (cf. Bondi & Meyer, 1943). The aim of the experiments described in this paper was, therefore, to investigate the influence of various hydrolytic treatments of the starting materials used for the preparation of alkali-extractable lignins and of the isolated lignins on their composition. Furthermore, an attempt was made, by studying methylation of the lignins with diazomethane and dimethyl sulphate, to draw conclusions with regard to the structure of the lignin building unit.

EXPERIMENTAL

Materials

Barley. Barley was harvested at the age of 2·5 months and dried in air. The hay was used for lignin extraction, and part of it was fed to sheep as their only food. Their faeces were collected and lignin was extracted from them by the same methods as from barley.

De-lintered cotton-seed hulls. In this case, lignin could be prepared only from the undigested material, since it proved impossible to maintain sheep on rations containing only cotton-seed hulls.

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Methods

The extraction and purification of lignins extracted by alkali, their methylation with diazomethane and dimethyl sulphate and the method of methoxyl determination have been described previously (Bondi & Meyer, 1948). Acid-processed lignins were prepared by the method of Kalb (1932) by treating the starting materials with a mixture of HCl and H_2SO_4. Samples of 1 g. were used throughout, since lignin prepared by the acid method in larger batches often showed marked inhomogeneity and the batches differed widely in composition.

Hydrolytic treatment of the starting materials

(*a*) *With dilute* H_2SO_4. 300 g. plant or faecal material (finely ground and passing a 0·5 mm. sieve) were suspended in 200 ml. of 0·5 N-H_2SO_4 and heated under reflux, with stirring for 6 hr. The mixture was then filtered through cheesecloth and washed free from acid with hot water. The residue was dried in air and used for the preparation of alkali-extractable lignin.

(*b*) *By heating with water under pressure.* Finely ground plant or faecal material (300 g.) was suspended in 2 l. of water and autoclaved at 210° for 2 hr. The mixture was filtered through cheesecloth, washed with 6 l. of hot water on the filter and the residue was dried and used for the preparation of alkali-extractable lignin. (Attempts to hydrolyse plant and faecal material with N- or 2N-acid at 210° failed owing to extensive decomposition and charring of the materials together with the formation of ether-soluble degradation products, mostly phenols, presumably from the lignin.)

Hydrolytic treatment of the isolated lignins

(*a*) *By heating with dilute* H_2SO_4. Isolated lignins remain unchanged when refluxed with 0·5 N-acid; they were therefore treated with the acid at higher temperatures in the following manner: lignin (15 g.) was suspended in 200 ml. of 0·5 N-H_2SO_4 and heated for 1 hr. at 150° in a glass-lined autoclave. After cooling the lignin residue was filtered off, washed free from acid and dried in air.

(*b*) *By heating with water.* Lignin (20 g.) was suspended in 200 ml. of water and autoclaved at 210° for 2 hr. The lignin residue was filtered off, washed free from acid and dried.

Degradation reactions

Treatment of the lignin with alkali under pressure. Lignin (20 g.) was dissolved in a mixture of 200 ml. of water and 50 ml. of 12N-NaOH and autoclaved at 155° for 2 hr. The temperature was then raised to 210° and kept there for 1 hr. After that the reaction mixture was allowed to cool. The solution was then acidified with 20% (v/v) H_2SO_4 to precipitate the unreacted lignin. Both the solution and the precipitate were exhaustively extracted with ether, the ether solution was dried with Na_2SO_4 and evaporated. The residue was weighed, redissolved in ether and separated into phenolic, acidic, aldehydic, and neutral fractions by methods previously described (Bondi & Meyer, 1948). The unreacted, ether-extracted lignin was suspended in 100 ml. of water, boiled for 2 min. filtered off, washed with water and dried.

RESULTS

The original nitrogen contents and the methoxyl contents of the lignins before and after methylation with diazomethane and dimethyl sulphate are given in Table 1.

Lignins from different plant sources. The elementary analyses of the three lignins investigated (see Table 2) do not show any significant differences between them, apart from the lower nitrogen content of the cotton-seed-hull lignin.

Cotton-seed lignin has also a lower initial methoxyl content than either lignin derived from barley (cf. Smith & Purves, 1944). After methylation, however, cotton-seed-hull lignin shows approximately the same values as barley lignin. The conclusion seems therefore justified, that cotton-seed-hull lignin does not differ from the others in its number of hydroxyl groups, but only in the degree of methylation.

Table 1. *Methoxyl and nitrogen content of the lignins as percentage of dry, ash-free material*

		Treatment			OMe*		
No.	Source	Starting material	Isolated lignin	N (%)	(1) (%)	(2) (%)	(3) (%)
1	Barley	Standard 'alkali lignin'	—	1·4	8·7	20·3	24·3
2		Dilute acid	—	1·5	6·3	15·8	18·1
3		Water, 210°	—	1·6	4·7	13·6	16·8
4		—	Dil. acid	1·4	7·0	17·3	21·9
5		—	Water, 210°	1·6	7·2	13·4	16·2
6		—	Alkali, 210°	1·3	7·4	14·6	19·1
7		—	KIO_4	1·9	4·4	13·7	13·9
8		—	Conc. acid	1·5	8·6	20·2	24·0
9		Conc. acid (acid-process lignin)	—	0·6	4·3	7·6	14·9
11	Faeces	Standard 'alkali lignin'	—	1·4	8·5	20·6	21·0
12		Dilute acid	—	1·5	6·6	16·5	19·0
13		Water, 210°	—	1·6	4·4	14·1	16·8
14		—	Dil. acid	1·4	8·0	16·1	20·4
15		—	Water, 210°	1·6	7·8	15·0	17·0
16		—	Alkali, 210°	1·3	6·8	19·3	19·6
17		—	KIO_4	2·6	4·3	14·0	14·3
18		—	Conc. acid	1·4	8·5	20·4	21·0
19		Conc. acid (acid-process lignin)	—	0·9	1·6	7·6	14·0
21	Cotton-seed hulls	Standard 'alkali lignin'	—	0·8	6·0	20·5	24·2
22		Dilute acid	—	0·8	5·1	15·3	19·5
23		Water, 210°	—	1·0	4·3	13·5	15·2
24		—	Dil. acid	0·7	5·2	18·4	21·5
25		—	Water, 210°	0·8	5·8	14·9	19·2
26		—	Alkali, 210°	0·8	5·0	14·8	18·7
27		—	KIO_4	1·2	4·4	13·8	14·0
28		—	Conc. acid	0·8	5·9	20·0	24·1
29		Conc. acid (acid-process lignin)	—	0·2	1·2	5·1	14·3

* Methoxyl contents: (1) initial; (2) after methylation with diazomethane; (3) after methylation with dimethyl sulphate.

The nitrogen content of the lignins. As in the lignins investigated before (Bondi & Meyer, 1948), no treatment could produce nitrogen-free lignins.

Table 2. *Elementary composition of lignins (in percentage of dry, ash-free material)*

Source	N	C	H	O
Barley (1)	1·40	63·3	5·7	29·6
Faeces (11)	1·40	63·8	5·2	29·6
Cotton-seed hulls (21)	0·80	63·6	5·4	30·2

Some treatments, like that with water at 210°, even raised the nitrogen content of the lignins, probably by attacking preferentially the nitrogen-free part

of the molecule. We therefore feel justified in upholding our previous contention that the nitrogen content of the lignins is not due to contamination with proteins and that nitrogen atoms form an integral part of the lignin molecule. Man & Heus (1950) have described lignins containing nitrogen and yielding amino-acids on hydrolysis (cf. also Thomas & Armstrong, 1949). We have often observed this phenomenon in impure lignins which initially contained 2·5–3·0% of nitrogen. When these were hydrolysed under conditions usually employed for protein hydrolysis, amino-acids could be found among the hydrolysis products, and determined quantitatively by the Sørensen and Pope-Stevens methods. When lignins are repeatedly dissolved and reprecipitated from acid, the initially high nitrogen content falls until it attains a constant value which cannot be lowered by any hydrolytic treatment. When this state is reached, no amino-acids can be found among the hydrolysis products. All nitrogen values reported in this paper are such end values. The lignin prepared by Man & Heus, by our method, which contained 8·8% nitrogen, seems to have been a preparation grossly contaminated with protein.

Acid-process lignins

Lignins obtained by the treatment of plant materials with concentrated acid differ considerably both physically and chemically from the alkali-extractable lignins. In contrast to the light brown, alkali- and ethanol-soluble 'alkali lignins', 'acid lignins' are black substances, insoluble in ethanol and only slightly soluble in dilute alkali. Very often 'acid lignins' still show the structure of the plant material from which they have been prepared. 'Alkali lignins' are generally obtained in yields of 3–7%, whereas yields of 'acid lignin' are much higher, in general 9–20%. The methoxyl and nitrogen contents are much lower than those of 'alkali lignins' prepared from the same starting materials, with the exeption of the methoxyl value obtained after methylation with dimethyl sulphate, which remains comparatively high. The reason is probably that 'acid lignins' are condensation products of the lignins proper with carbohydrate materials (Norman & Jenkins, 1934) probably hemicelluloses which contain no nitrogen or methoxyl, but contribute a number of alcoholic hydroxyl groups. Condensation between lignin and hemicellulose affects all the free hydroxyl groups of the lignin, thereby abolishing the solubility in ethanol and alkali. The high lignin yields obtained by treating plant tissues with concentrated acids can therefore give no measure of the true lignin content. The assumption that condensation reactions take place during acid treatment was tested by treating plant materials from which the lignin had already been extracted by alkali with 72% (v/v) sulphuric acid and with hydrochloric-sulphuric acid mixture, according to the method of Kalb (1932). Black substances were obtained which were in appearance indistinguishable from 'acid lignins' but which contained no methoxyl and gave, on degradation with nitrobenzene and alkali, no vanillin, a substance given by the oxidative degradation of all true lignins. Isolated lignins are, however, not affected by treating them with cold concentrated acids. (Compare lignins nos. 8, 18 and 28 in Table 1 with lignins nos. 1, 11 and 21.) Similar results were reported by Kudzin & Nord (1951). It is our opinion that, in order to obtain reliable analytical lignin determinations, carbohydrates, especially hemicelluloses, must be removed as completely as possible from plant tissues before they are subjected to the influence of strong acids.

The influence of the various hydrolytic treatments on the lignins

We have reported previously that dilute alkali extracts a lignin-hemicellulose complex which is easily hydrolysed by dilute acid. This observation, together with the high initial methoxyl values of the 'alkali lignins' (obtained without any treatment of the starting materials prior to the lignin extraction), shows that alkali extraction is one of the mildest methods for lignin isolation, since it leaves even the weak lignin-hemicellulose bonds and acetalic methyl groups intact. We therefore regard the 'alkali lignins' as comparatively unchanged (lignins nos. 1, 11 and 21 in Table 1) and refer to them as 'standard lignins'.

The changes brought about by the various treatments both of the starting materials and of the isolated lignins seem all to affect hydroxyl groups. When plant materials are subjected to acid treatment (or water at high temperatures, which has a similar effect) the lignin-hemicellulose bonds open and hydroxyl groups previously blocked are set free.

Both acid and high temperatures seem to favour condensation reactions involving the hydroxyl groups previously set free. Consequently all lignins prepared from pre-treated starting materials have fewer free hydroxyl groups than those prepared from untreated starting materials. This is shown by the fact that all methoxyl values obtained after methylation are always lower than the corresponding values in standard lignins. Diminution of the number of free hydroxyl groups in lignin is effected by all treatments, and the differences between the various treatments seem to be in degree rather than in kind. Mere digestion in the animal body influences mainly side-chain (aliphatic) hydroxyl groups (which are methylated by dimethyl sulphate only). In faeces lignin, therefore, the differences between the methoxyl values obtained after methylation with diazomethane and dimethyl

sulphate found in barley lignin (lignin no. 1), have almost entirely disappeared. This effect had previously been observed in a number of plant materials (cf. Bondi & Meyer, 1948). When, however, the starting materials were pretreated with acid or water at high temperature, both aliphatic and aromatic hydroxyl groups take part in condensation reactions, consequently both the diazomethane and dimethyl-sulphate methylation values of all lignins prepared from pretreated starting materials are lowered considerably. In addition, pretreatment of the starting materials removes part of the aliphatic methyl groups originally present, here the influence of water at high temperatures is greater than that of acids. Disappearance of free hydroxyl groups was the main effect caused by the treatment of isolated lignins with the different hydrolysing agents, hydrolysis with water at 210° being the most effective. It lowered both the diazomethane and the dimethyl-sulphate methylation values more than any other treatment.

It has been assumed that condensation reactions are responsible for the lowering of methoxyl values obtainable by methylation of the lignins subjected to the different treatments. This assumption was preferred to the alternative one that hydrolysis splits off both aliphatic and aromatic hydroxyl groups. The splitting off of aromatic hydroxyl is, under the conditions employed by us, a very improbable reaction, and the splitting off of side chains would inevitably lead to the formation of low-molecular degradation products, since it is generally assumed that by condensation reactions between these side chains the peculiar structure of the lignin molecule is achieved.

The assumption of condensation reactions between hydroxyl groups leading to the formation of C—O—C bonds was tested by treating the lignins with hydriodic acid, which, by opening the bonds, would lead to the reappearance of hydroxyl groups. The lignins were refluxed in 2 g. portions with 20 ml. hydriodic acid solution (sp.gr. 1·71) for 4 hr. The residual lignins were filtered off, washed and dried and methylated with dimethyl sulphate. Table 3 shows that in every case products richer in methoxyl were obtained by methylation of lignins treated with hydriodic acid, than by the same methylation of the original lignins. Additional hydroxyl groups have therefore appeared as a result of the hydriodic acid treatment.

No increase in methoxyl could, however, be obtained when lignins were oxidized with potassium periodate and afterwards treated with hydriodic acid. Periodate oxidation apparently leads in the case of the lignin to the oxidative removal of side chains containing both hydroxyl and methoxyl groups (see Wald, Ritchie & Purves, 1947). As a result, lignins so treated have no more condensed hydroxyl groups which can be set free by hydriodic acid treatment. Furthermore, the initial methoxyl content is lowered to approximately one half its former value owing to the loss of side-chain methoxyl and the diazomethane methylation value

Table 3. *Methoxyl values after methylation with dimethyl sulphate of lignins treated with hydriodic acid*

Lignin no.	OMe (after methylation with dimethyl sulphate)	OMe (after treatment with HI and subsequent methylation with dimethyl sulphate)
3	16·8	21·4
13	16·8	21·5
23	15·2	21·3
5	16·2	20·9
15	17·0	21·0
25	19·2	21·8
7	13·9	14·3
17	14·3	14·3
27	14·0	14·4

approaches a limiting value between 13 and 14%, which is in all probability attributable to aromatic hydroxyl.

Degradation reactions

Potassium periodate oxidation led to the formation of formaldehyde, as was to be expected. Formaldehyde was identified by its reaction with chromotropic acid and its dimedone derivative, but formic acid could not be detected. Degradation of the lignins by heating them with alkali under pressure led to the formation of aromatic degradation products which were separated into an aldehydic, acidic, phenolic and neutral fractions by methods previously described (Bondi & Meyer, 1948). By repeated alkali treatment the yield of aromatic degradation products could be increased to about 25%. No new degradation products were identified.

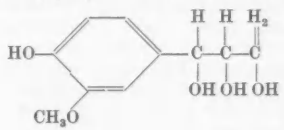
The structure of the lignins

That the phenylpropane unit (C_6–C_3) forms the backbone of the wood-lignin building units seems now to be well established by the investigations of Hibbert (1942) and Freudenberg (cf. Freudenberg, Sohns & Janson, 1935). Support is also given by the work of Phillips & Goss (1932) and of ourselves (Bondi & Meyer, 1948). The experimental results presented in this paper will therefore be discussed under the assumption that the basic lignin structural unit is a phenylpropane derivative, substituted in the ring by OH and/or CH_3 groups and containing in its side chain alcoholic and enolic OH groups and probably also aldehyde or keto groups (cf. Brauns, 1939).

One half of the initial methoxyl content of the lignins is apparently contained in its side chains, since it is removed by potassium periodate oxida-

tion, the remainder is aromatic, as appears from the presence of vanillin among the lignin degradation products. The side-chain methoxyl is probably acetalic, since it is resistant to alkaline hydrolysis, but it is easily split off by acid. Since it was previously found (Bondi & Meyer, 1948) that every lignin building unit contains two methoxyl groups and yields vanillin on degradation, the total number of methoxyl groups initially present would be four, two aromatic and two aliphatic. (In cotton-seed lignin only three methoxyl groups are present, the fourth being unsubstituted OH.) The average percentage of initial methoxyl is 8·6 (lignins nos. 1 and 11) thus making the molecular weight of a building unit 1430, and the empirical formula $C_{76}H_{76}O_{26}N$. Deducting the four carbon atoms of the methoxyl groups leaves 72 carbon atoms, or eight C_6–C_3 units. The average value of 20·4 % methoxyl after methylation with diazomethane accounts for six additional (aromatic and enolic) hydroxyl groups. The increase in methoxyl after methylation with dimethyl sulphate to 24·25 % (average value of lignins nos. 1 and 21) corresponds to the presence of two additional (aliphatic) hydroxyl groups. The eight phenylpropane units forming the lignin building unit are supposed to be subdivided into units of four, connected by a nitrogen instead of an oxygen bridge, but not regularly, since the nitrogen content of the various lignins is more variable than that of the other constituents. Depolymerization of the lignin molecule seems to take place preferably at the nitrogen bridges, which explains the lower molecular weights found by us by cryoscopic determination in naphthol, which is a depolymerizing agent.

If the phenylpropane unit has the structure



according to Freudenberg's theory, it will follow that every such unit must be connected to the others by at least three oxygen bridges in order to correspond to the formula proposed in this paper. This would explain the extraordinary stability of the lignin molecule, which can be destroyed only by very strong reducing agents at high temperatures, such as hydriodic acid and phosphorus under pressure (cf. Willstaetter & Kalb, 1922). The exact mode of connexion between the phenylpropane units must, however, await further experimental elucidation.

SUMMARY

1. Alkali-extractable lignins ('alkali lignins') were prepared from barley, from faeces collected from sheep fed exclusively with barley, and from cotton-seed hulls.

2. The materials used for lignin extraction and the isolated lignins were subjected to hydrolysis with dilute acid, water at 210° and alkali at 210°.

3. From determinations of the initial methoxyl content and of the methoxyl content after methylation with diazomethane and with dimethyl sulphate, of all the lignins, the following conclusions were drawn:

(*a*) All treatments with acid, including the conventional lignin determination methods, and treatment involving high temperatures, lead to extensive condensation reactions within the lignin molecule, through the aliphatic and aromatic hydroxyl groups.

(*b*) Treatment with dilute alkali leaves lignin comparatively unchanged, treatment with stronger alkali and at temperatures over 100° leads to their gradual degradation.

4. The nitrogen content of the lignins is not due to their contamination with protein.

5. The lignins contained in the plants investigated are very similar, probably all containing the same phenylpropane basic structural unit. The structural details of the lignin molecule have been discussed in the light of the results obtained.

REFERENCES

Bondi, A. & Meyer, H. (1943). *J. agric. Sci.* **33**, 123.
Bondi, A. & Meyer, H. (1948). *Biochem. J.* **43**, 248.
Brauns, F. E. (1939). *J. Amer. chem. Soc.* **61**, 212.
Freudenberg, K., Sohns, F. & Janson, A. (1935). *Liebigs Ann.* **518**, 76.
Hibbert, H. (1942). *Ann. Rev. Biochem.* **11**, 183.
Kalb, L. (1932). In Klein, G., *Hanbuch der Pflanzenanalyse*, **3**, 190.
Kudzin, S. F. & Nord, F. F. (1951). *J. Amer. chem. Soc.* **73**, 690.
Man, Th. J. de & Heus, J. G. de (1950). *Rec. trav. chim. Pays-Bas.* **69**, 271.
Norman, A. G. & Jenkins, S. H. (1934). *Biochem. J.* **28**, 2173.
Phillips, M. & Goss, M. (1932). *Industr. Engng Chem.* **24**, 1936.
Smith, M. A. & Purves, C. B. (1944). *Industr. Engng Chem.* (Anal. ed.), **13**, 157.
Thomas, B. & Armstrong, D. G. (1949). *J. agric. Sci.* **39**, 335.
Wald, W. I., Ritchie, P. F. & Purves, C. B. (1947). *J. Amer. chem. Soc.* **63**, 1371.
Willstaetter, R. & Kalb, L. (1922). *Ber. dtsch. chem. Ges.* **55**, 2637.

The Estimation of Serum Bilirubin

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The more important methods used and the problems involved in the estimation of bilirubin in serum have been adequately reviewed in recent times by Lemberg & Legge (1949) and by King & Coxon (1950). The methods are of two types: those based essentially on the original van den Bergh technique for the so-called 'indirect reaction' (Thannhauser & Andersen, 1921; Haslewood & King, 1937), and those which promote coupling between van den Bergh reagent and bilirubin without precipitation of proteins. In the latter type, special catalysts or accelerators are employed, e.g. sodium benzoate and caffeine (Jendrassik & Cleghorn, 1936), urea and caffeine (Rappaport & Eichhorn, 1943), thereby avoiding the use of alcohol or other protein precipitants. Alternatively, alcohol is used in relatively low concentration in the presence of diluted serum in such a way that no precipitation occurs (Malloy & Evelyn, 1937).

The main defect of the first type of method lies in the fact that, in the course of protein precipitation and its subsequent removal, considerable loss of either bilirubin or azobilirubin is incurred. On the other hand, methods of the second class suffer from the fact that the clarity of the final coloured solutions leaves much to be desired. In some cases, indirect bilirubin fails to react completely in a reasonable space of time (Gray & Whidborne, 1946). King & Coxon (1950), dissatisfied with the performance of the non-precipitation methods, have reverted to the original type of procedure with a modification which employs a higher dilution of serum and reagents in the presence of ethanol than is customary, thereby minimizing the loss of azobilirubin.

We have investigated the van den Bergh reaction to see whether we could arrive at conditions whereby real clarity could be achieved in the final solution prepared for colorimetric analysis without recourse to any step involving separation of proteins, and at the same time utilize a reaction which would proceed rapidly to completion in all types of case, no matter what the form of bilirubin present.

Rapid coupling of indirect bilirubin with the diazo reagent is achieved by use of urea-ethanol solutions in conditions which introduce only slight opacity, representing a very small degree of protein precipitation. This cloudiness completely disappears when concentrated phenol solution is added. Employment of phenol in connexion with the development of a diazo colour requires considerable care. Regard must be paid to the necessity of inactivating the excess of diazo reagent before the phenol is added, and also to avoidance of the formation of any pink oxidation product from the phenol used.

Following the observation of Lees (1950) that azide would react with the diazonium component of the Griess-Ilosva reagent to diminish the colour intensity produced by subsequent coupling with 1-naphthylamine, azide was tried as a simple means of inactivating excess of the van den Bergh reagent and was found highly effective. The addition, therefore, of a trace of azide, and the removal of excess of nitrous acid by the usual sulphamate addition, overcome the objections to the introduction of phenol into the van den Bergh reactions, and allows the completion of a method which in practice has proved satisfactory in its application to all clinical cases so far investigated.

METHOD

Reagents

Diazo reagent. Solution A, 1 g. of sulphanilic acid in 1 l. 0·175 N-HCl. *Solution B*, 0·5 g. $NaNO_2$ in 100 ml. of aqueous solution. 10 ml. of *A* are mixed with 0·3 ml. of *B*. After 5 min. 1 ml. of 1·5% (w/v) ammonium sulphamate is added, and the reagent used after standing a minimum of 3 min. A corresponding 'blank' reagent is made by substituting distilled water for solution *B* in the dizao reagent.

Ethanol-urea solution. With the aid of heat, 25 g. of urea (A.R.) are dissolved in 100 ml. of 85% (v/v) ethanol, filtered while still warm and kept in an incubator to prevent crystallization of urea.

Sodium azide. Solid.

Phenol solution. To 90 parts by weight of freshly redistilled pure phenol are added 10 parts of water with stirring.

Acid buffer solution. 29·41 g. of sodium citrate ($Na_3.C_6H_5O_7.2H_2O$ A.R.) are dissolved in 0·2 N-HCl to a volume of 500 ml. 200 ml. of this buffer are made up to 500 ml. with distilled water.

Neutral buffer solution. Sodium citrate (5·0 g. A.R.) is dissolved in distilled water and made up to 100 ml.

Procedure

In each of two glass-stoppered test tubes of 10 ml. capacity place 0·5 ml. of serum. To the first add 0·5 ml. of van den Bergh diazo reagent-sulphamate solution, and to the second, add 0·5 ml. of the corresponding blank reagent. Place both tubes in a beaker containing ice-cooled water.

Add to each 2·2 ml. of ethanol-urea solution, followed by 0·2 ml. of $CHCl_3$. Mix and keep ice-cooled for 10 min. Add a tiny fragment of azide (about 0·2 mg.) to each tube. Shake to dissolve, and then add 0·1 ml. of neutral citrate buffer and mix. Finally add 2·5 ml. of the phenol solution. Stopper each tube securely and shake vigorously until a clear solution is obtained. Read the van den Bergh colour against that produced by the blank reagent in the photoelectric colorimeter using a green filter. (In routine work the Biochem absorptiometer, standard test tubes, and a Chance green filter OG 1 (maximum transmission 520–540 mμ.) have been used, but the data obtained have been further substantiated by the supplementary use of the Spekker absorptiometer with Ilford filter 604.)

Where the serum bilirubin exceeds 8 mg./100 ml. the determination is repeated using serum suitably diluted with acid buffer.

Calibration

Pure bilirubin (Hoffman-La Roche) was used as the standard preparation for calibration purposes. Compared with other readily available commercial samples, it had a higher chromogenic power, which could not be increased by attempts at further purification using chromatographic methods and recrystallization. Moreover, it was previously used in this type of work and found satisfactory by earlier workers (Malloy & Evelyn, 1937; Gray & Whidborne, 1946).

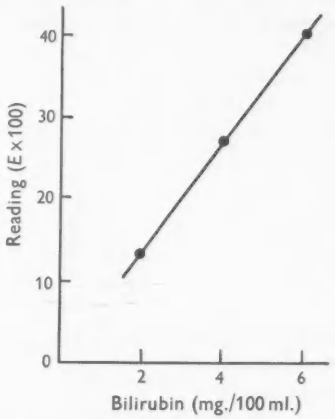

Fig. 1. Calibration curve. Spekker absorptiometer with Ilford filter 604 and 1 cm. cells.

Pure bilirubin (10 mg.) was dissolved in 100 ml. of $CHCl_3$, and graded dilutions in $CHCl_3$ were prepared from this stock.

The technique used in calibration was exactly the same as for the test method, except for using 0·2 ml. of bilirubin solution in place of the pure $CHCl_3$ and acid citrate buffer in place of serum. Fig. 1 shows the relation between colour density and concentration of bilirubin. Because of the sensitivity of the azobilirubin colour to changes in pH, adequate control of this factor is only assured if the colour developed in the calibration technique can be accurately identified with that produced in the test method. Figs. 2 and 3 record the spectral absorption curves obtained, respectively, from pure bilirubin and from a jaundiced serum of approximately equivalent bilirubin concentration, using on the one hand a water blank and on the other a yellow blank prepared as indicated in the above procedure. It is shown that a close identity exists between the corresponding curves of these two charts.

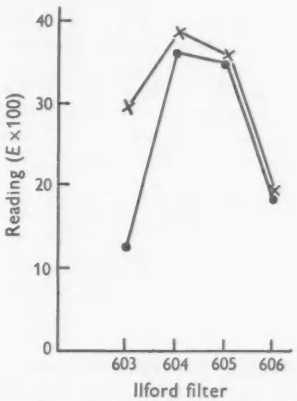

Fig. 2. Spectral absorption curves of azobilirubin colours produced in the calibration method from pure bilirubin. ×—×, azobilirubin read against a colourless blank; ●—●, azobilirubin read against the corresponding yellow blank. Spekker absorptiometer with Ilford filter 604.

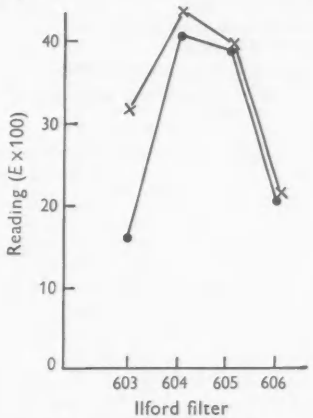

Fig. 3. Spectral absorption curves of azobilirubin colours produced from serum bilirubin. ×—×, azobilirubin read against a colourless blank; ●—●, azobilirubin read against the corresponding yellow blank. Spekker absorptiometer with Ilford filter 604.

RESULTS

The reproducibility of results with sera was examined by the performance of a series of five estimations on an individual sample. The following values by the routine method were obtained: 3·32, 3·25,

3·36, 3·25, 3·32 mg. bilirubin/100 ml., giving a mean value of 3·30 mg./100 ml. and a standard deviation from the mean of 0·03 mg./100 ml.

Time factor in colour development. In order to determine the time required for full development of colour under the conditions of the test, a number of

Table 1. *Recovery of bilirubin added to sera*

(Zero values in first column indicate <0·1.)

Serum bilirubin (mg./100 ml.)	Bilirubin added (mg./100 ml.)	Total bilirubin found (mg./100 ml.)	Added bilirubin recovered (mg./100 ml.)	Recovery (%)
Readings on Biochem absorptiometer, Chance filter OG 1				
0	3·67	3·80	—	104
0	4·54	4·37	—	97
0	6·05	5·83	—	96
0	7·64	7·36	—	96
0·55	4·00	4·62	4·07	102
1·08	4·00	5·20	4·12	103
0·60	3·67	4·31	3·71	101
Readings on Spekker absorptiometer, Ilford filter 604				
0	3·55	3·56	—	100
0	3·67	3·83	—	104
0·40	3·67	4·00	3·60	98

Table 2. *Serum bilirubin values (mg./100 ml.) derived from azobilirubin colour produced after varying reaction times*

(Readings on Spekker absorptiometer, Ilford filter 604.)

Type of serum	Reaction time (min.) 2	5	10	20	45
Cord-blood serum from jaundiced baby; indirect reacting	—	—	8·6	8·7	—
Toxic hepatitis; latent jaundice; indirect reacting	—	—	1·80	1·80	—
Infective hepatitis; direct reacting	6·0	6·4	6·2	6·4	—
Cord-blood serum from jaundiced baby; indirect reacting	—	—	6·1	6·1	6·1

Table 3. *Serum bilirubin estimated at varying dilutions using acid citrate buffer and serum (of negligible bilirubin content) as diluents*

(Readings made with Biochem absorptiometer.)

	Diluted with citrate		Diluted with serum	
Type of serum	Dilution	Bilirubin (mg./100 ml.)	Dilution	Bilirubin (mg./100 ml.)
Obstructive jaundice	1 in 4	23·5	1 in 4	24·9
	1 in 6	24·4	1 in 6	24·0
	1 in 8	24·8	1 in 8	23·7
Toxic jaundice	1 in 2	2·86	—	—
	Undiluted	2·75	—	—
Obstructive jaundice	1 in 2	7·04	—	—
	1 in 4	6·73	—	—

Recoveries. The percentage recovery was estimated by adding known amounts of bilirubin in the form of chloroform solution to the serum mixture. This addition can be done at any stage up to and including the application of the urea-ethanol solution. Selected sera containing negligible amounts of bilirubin (less than 0·1 mg./100 ml.), and also normal sera, were used. The results are shown in Table 1.

estimations on different types of jaundiced sera were made, allowing varying intervals between the addition of the urea-ethanol and that of the azide. Table 2 records some typical results, which show that a 10 min. period is adequate for the full reaction to take place.

A number of jaundiced sera were examined, using as diluents the citrate buffer on the one hand and serum containing a negligible amount of bilirubin on

the other. The data in Table 3 show that concordant estimations are obtained at widely differing dilutions, and that the buffer does not introduce error in the final values when replacing serum.

Analytical data are presented in Tables 4 and 5 to show the general relationship of the results obtained

Table 4. *Estimations of bilirubin in direct sera*

(Indices indicate dilution factors. *A*, read on Biochem absorptiometer, Chance filter OG 1; *B*, read on Spekker absorptiometer, Ilford filter 604, except for the Malloy & Evelyn (1937) method when 605 filter was used. The calibration curve used for the Malloy & Evelyn determinations was constructed as described by the original authors with the exception that methanol was used in place of ethanol. On account of the different effects of the two alcohols upon the azobilirubin colour tone, the colour of the calibration solutions is not so accurately reproduced in those of the test method if the same alcohol is not used for both.)

Case	Present method (mg./100 ml.)	Malloy & Evelyn (1937) method (mg./100 ml.)	King & Coxon (1950) method (mg./100 ml.)
A			
1	$22{\cdot}8^{6}$	—	$19{\cdot}6^{4}$
2	$22{\cdot}4^{4}$	$24{\cdot}1^{2}$	$22{\cdot}0^{4}$
3	$22{\cdot}0^{4}$	$21{\cdot}8^{2}$	$18{\cdot}4^{4}$
4	$16{\cdot}1^{4}$	16·0	—
5	$11{\cdot}4^{2}$	—	$10{\cdot}2^{2}$
6	$11{\cdot}0^{2}$	11·1	$8{\cdot}8^{2}$
7	$10{\cdot}6^{2}$	11·8	$8{\cdot}2^{2}$
8	$7{\cdot}8^{2}$	—	7·8
9	6·3	—	6·6
10	5·2	5·2	4·4
11	3·2	—	2·0
12	3·0	3·0	2·0
B			
13	$24{\cdot}0^{3}$	—	$20{\cdot}8^{3}$
14	$21{\cdot}8^{4}$	$21{\cdot}0^{2}$	$15{\cdot}6^{4}$
15	$19{\cdot}9^{3}$	$21{\cdot}6^{2}$	$15{\cdot}9^{3}$
16	$17{\cdot}1^{2}$	—	$12{\cdot}8^{2}$
17	$16{\cdot}3^{4}$	15·2	$14{\cdot}7^{4}$
18	7·1	7·2	5·5
19	4·2	—	3·3
20	3·7	—	3·2
21	2·2	—	1·5
22	1·7	—	1·3

by the present method, that of Malloy & Evelyn (1937) as representing one of the most widely used of the non-precipitation methods, and that of King & Coxon (1950) as an example of a recent type of precipitation technique.

DISCUSSION

The technical procedure calls for a brief comment upon the choice of reagents. Urea-ethanol is used in place of ethanol alone in developing the total reactivity of the bilirubin present for two reasons. Urea itself has been shown by Gray & Whidborne (1947) to have an accelerating action upon indirect bilirubin, and it can be safely used up to the concentration employed in the present method, though greater concentrations impede the reaction. Moreover, the denaturation effect on the serum protein of urea-ethanol is different from that of ethanol alone, for any precipitated protein formed at that stage is more readily dissolved in the phenol solution used later. The ethanolic concentration present at the colour-development stage is well in excess of 50 % and therefore adequate according to our own experience and that of Malloy & Evelyn as to the requisite minimum ethanol concentration for full colour production. In all cases that have been investigated a 10-min. period has been found ample for maximum colour development.

Table 5. *Estimations of bilirubin in 'indirect' and 'delayed direct' sera*

(Indices indicate dilution factors. *A*, read on Biochem absorptiometer, Chance filter OG 1; *B*, read on Spekker absorptiometer, Ilford filter 604, except for the Malloy & Evelyn (1937) method when 605 filter was used.)

Case	Present method (mg./100 ml.)	Malloy & Evelyn (1937) method (mg./100 ml.)	King & Coxon (1950) method (mg./100 ml.)
	Series *A*		
1	1·82	—	2·00
2	1·10	—	1·20
3	0·83	—	0·80
4	0·69	—	0·70
Horse serum 1	3·39	—	3·10
Horse serum 2	1·32	—	1·14
	Series *B*		
5	$23{\cdot}7^{4}$	22·7	$20{\cdot}6^{4}$
6	$10{\cdot}2^{2}$	10·6	$9{\cdot}3^{2}$
7	7·4	7·8	7·0
8	6·1	6·6	5·5
9	2·55	—	2·64
10	1·04	—	0·82
11	0·69	—	0·59

The neutral citrate solution added prior to the phenol solution could be omitted in dealing with the great majority of sera, but, in its absence, some specimens of exceptionally low buffering capacity tended to give colours of a slightly bluer tint than did normally buffered sera, and the results were, therefore, rendered less reliable. Similarly, the addition of the pure chloroform might also be considered dispensable in a general way. It has been retained throughout, not only because it accurately balances in the estimation the conditions of the calibration method, but because, with specimens that are lipaemic, its presence gives a rather brighter final solution.

In that strong phenol solutions require special care in handling, alternative reagents that have a marked solvent effect upon protein, e.g. concentrated thiocyanate and aqueous urea, have been examined, but have been found less effective in providing high clarity than phenol.

Methanol has also been investigated as an alternative to ethanol in the urea-ethanol solution. As in

the Malloy & Evelyn (1937) method it gives a rather greater amount of colour for a given concentration of bilirubin (Moreland, O'Donnell & Gast, 1950) and rather less protein precipitation in the colour development stage, but offers no advantage in reaching final clarity.

While serum is preferred for this form of analysis, plasma can be used with almost the same precision in the estimations. There is a tendency for the final solution in the case of plasma to have a very slightly lower degree of optical clarity. This is balanced by the corresponding blank solution, so that the colorimeter readings are not affected, and in general the solutions with plasma are clearer than those obtained by other non-precipitation techniques which use only serum.

In regard to the comparative results of the three methods employed, it is scarcely to be expected that they should provide identical results. Although the Malloy & Evelyn method and the present method are fundamentally the same in that neither entails loss by separation of protein, the calibration method is a little different in the two cases. Malloy & Evelyn standardize with pure bilirubin solutions without relation to blanks incorporating unchanged bilirubin, whereas the unchanged pigment necessarily appears in the blanks corresponding to the serum tests. In the present method, unchanged bilirubin appears in both the blank of the standardization technique and that of the test.

The King & Coxon method does not involve any blank preparation either in calibration or in serum analysis, for the precipitation of protein takes with it any constituent likely to give an appreciable degree of light absorption other than that due to azobilirubin. In a blank designed to run parallel with their rest method, a high proportion of unchanged bilirubin is absorbed on the protein precipitate, so there cannot in practice be a blank comparable to that employed in the present method.

These considerations would always account for minor differences in the values obtained by the different methods, but reference to Table 4 shows that, while closely comparable results are obtained between the present method and that of Malloy & Evelyn, the King & Coxon method often gives figures in the direct-reacting sera which lag substantially behind. They represent, however, a much nearer approach to the figures of the non-precipitation methods than those obtained by earlier precipitation techniques, for losses of up to 50% of colour were encountered in the method of Haslewood & King, when that was compared with the Malloy & Evelyn procedure (Gray & Whidborne, 1946). In the case of indirect-reacting sera, the results of King & Coxon and those of the present method are seen to be in closer agreement.

In that King & Coxon use an artificial standard of methyl red solution, whereas in the present method as well as in that of Malloy & Evelyn we have employed a preparation of bilirubin, reference should be made to the relationship between the two standards. Substituting in the King & Coxon technique 1 ml. of 3·6% $Na_2HPO_4 . 12H_2O$ for serum, adding to it 0·4 ml. of chloroform containing 0·04 mg. of bilirubin, then adding 0·5 ml. of their van der Bergh reagent, and finally making up to a volume of 10 ml. with 85% ethanol, we obtained an absorptiometer reading equal to 99% of that of the methyl red standard. With 1 ml. of our acid citrate buffer in place of serum and other reagents used as above, the reading was 101% of the same standard. We are therefore satisfied that we are attaching no unfair disadvantage to the recorded values for the King & Coxon method in assessing them in terms of their artificial standard solution.

The main advantages of the present method over that of Malloy & Evelyn are that the final colour is observed in clearer solution, and that, for a given concentration of serum bilirubin, the colours are much more intense, enabling more accurate readings to be made at much lower levels of bilirubin concentration. The method is, therefore, especially well suited to studies such as those of bilirubin threshold, and of the removal from the blood of injected bilirubin. In conjunction with appropriate precipitation methods, it is likely to facilitate further investigation into the cause of discrepancies which exist between the results of the two types of method of estimation. It may also be easily adapted to provide the means of following the rates of reaction of the different types of bilirubin present simultaneously in blood, and thereby contributed further to the understanding of the physico-chemical nature of the circulating bilirubin.

SUMMARY

1. A method is described for determining bilirubin in serum which does not involve separation of protein. Colour is developed by the van den Bergh reagent in the presence of a urea-ethanol solution, and clarity achieved by use of phenol, following the inactivation of excess diazonium compound.

2. The results achieved by the present method are in closer alinement with those of Malloy & Evelyn (1937) than with those of King & Coxon (1950).

3. As compared with the method of Malloy & Evelyn, the present technique is capable of giving greater accuracy at low bilirubin levels.

We are grateful to Dr P. L. Mollison, Director of the Medical Research Council Blood Transfusion Research Unit, and to Prof. E. J. King, of the Postgraduate Medical School of London, for placing at our disposal samples of cord blood of jaundiced infants.

REFERENCES

Gray, C. H. & Whidborne, J. (1946). *Biochem. J.* **40**, 81.
Gray, C. H. & Whidborne, J. (1947). *Biochem. J.* **41**, 155.
Haslewood, G. A. D. & King, E. J. (1937). *Biochem. J.* **31**, 920.
Jendrassik, L. & Cleghorn, R. H. (1936). *Biochem. Z.* **289**, 1.
King, E. J. & Coxon, R. V. (1950). *J. clin. Path.* **3**, 248.
Lees, H. (1950). *Biochem. J.* **47**, xliv.
Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments.* New York: Interscience.
Malloy, H. T. & Evelyn, K. A. (1937). *J. biol. Chem.* **119**, 481.
Moreland, F. B., O'Donnell, W. W. & Gast, J. H. (1950). *Fed. Proc.* **9**, 207.
Rappaport, F. & Eichhorn, F. (1943). *Lancet*, **1**, 62.
Thannhauser, J. S. & Andersen, E. (1921). *Dtsch. Arch. klin. Med.* **137**, 179.

Lipovitellin

2. THE INFLUENCE OF WATER ON THE STABILITY OF LIPOVITELLIN AND THE EFFECTS OF FREEZING AND OF DRYING

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The lipid-protein complexes (lipoproteins) of tissues are usually said to be decomposed by freezing or drying; in fact, modern theories of their structure generally include water as an integral part of the molecule (e.g. Dervichian, 1949). Little or no quantitative information on the subject has, however, been published and, in view of the increasing tendency towards preserving sensitive biological materials and foods in the frozen or dried state, an investigation of the effects on a lipoprotein of freezing and of drying has been carried out. Lipovitellin from egg yolk was chosen as experimental material for reasons already given (Lea & Hawke, 1951).

Available information on the stability of lipovitellin is conflicting. Chargaff (1942) stated that a suspension of the lipoprotein lost some of its dispersibility in sodium chloride solution on freeze-drying. Moran (1925) observed that a solution of 'lecithovitellin' in the minimum concentration of sodium chloride deposited solid on freezing and thawing. Levold & Lausten (1946) stated that 'lipovitellinin', a lipoprotein fraction from egg yolk containing 36–41 % lipid, was not, by freeze-drying, rendered insoluble in sodium chloride solution saturated with ether, but that its dispersibility disappeared on standing. No information was given as to whether or not any free lipid was split off from the protein during freezing, drying or storage, and in none of these cases was the moisture content of the dried material or the conditions of freezing and thawing defined, nor were any quantitative data recorded on the effects observed. It is, however, of considerable practical interest that commercial spray-dried egg, which has a greatly inferior aerating power to fresh egg, yields up much more of its fat on extraction with cold light petroleum than does fresh egg (Brooks & Hawthorne, 1944).

The present paper deals with some of the properties of lipovitellin in relation to the effects of freezing and drying and of storage in the frozen and dried states.

METHODS

Preparation of lipovitellin. Lipovitellin was prepared as previously described (Lea & Hawke, 1951) except that before the final dialysis any material which was not dispersible in 10% (w/v) NaCl saturated with ether was separated and discarded. It was found that if the final dialysis through cellulose at 0–2° was continued for longer than 24 hr. it became impossible, with the means available, to centrifuge down the suspended lipoprotein. The lipoprotein suspension was therefore centrifuged after dialysis for 24 hr. and the concentrated product subjected to continued dialysis with agitation from narrow cellulose sacks for a further 48 hr. at 0°.

In some preparations, 50% (w/v) sucrose was dissolved in the aqueous phase to protect the lipoprotein from denaturation by ether (Bate-Smith, 1935). This precaution could not be taken, however, in the first ether extraction of the yolk because of increased difficulty with emulsification, and was omitted from the final reprecipitation to avoid the lengthy dialysis necessary to remove the last traces of sugar from the thick lipoprotein suspension. Under these conditions, the use of sucrose resulted in no improvement in the yield or solubility of the preparation.

The lipid contents of lipovitellin preparations 18–29 used in this work were 27·5, 38·2, 38·2, 32·4, 34·2, 33·2, 27·5, 25·7, 28·4, 32·1, 31·5 and 30·2% respectively.

Determination of solubility. To 2 ml. lipoprotein suspension containing approximately 200 mg. lipoprotein were added 5 ml. NaCl of the strength necessary to give the required final concentration, 0·8 ml. ether and sufficient dilute NaOH or HCl and water to produce the desired pH and a final volume of 10·6 ml. The solutions were mixed by sudden shaking, slowly tumbled for 16 hr., centrifuged, and the N content of the supernatant fluid determined by the micro-Kjeldahl procedure. For most of the routine estimations of solubility 15% (w/v) NaCl and pH 6·0 were used. All operations were carried out at 0°. Although the solubilities of fresh preparations were usually in the range 93–97%, suggesting the possibility of incomplete equilibration or of denaturation during the determination, shorter or longer times of equilibration failed to give higher results than those obtained at 8 or 16 hr.

Determination of free lipid. The degree of decomposition of the lipoprotein was measured by estimating the amount of lipid extractable with ether. NaCl (500 mg.) was added to 5 ml. portions of the suspension containing approximately 500 mg. lipoprotein and the solution extracted at 0° by shaking in a 'Microid' wrist-action shaker with ether (100 ml.) saturated with water, usually for 15 min. The ether was then separated and its lipid content determined by drying on a rotary film evaporator (Lea, Hannan & Rhodes, 1951), taking up in dry ether, filtering and redrying, first under an infrared lamp and finally to constant weight *in vacuo* at 70°.

Lipoprotein samples which had been dried were shaken with dry ether for 30 min. at 0° or laboratory temperature, filtered, and the lipid content of the extract plus washings determined as above. In a second procedure, the dried samples (500 mg.) were re-moistened with 10% (w/v) NaCl (5 ml.) and extracted as for undried samples.

Freezing, thawing and storage in the frozen state. To investigate the effects of freezing, the lipovitellin suspension was frozen as a shallow layer in thin aluminium dishes by immersion in liquid at temperatures between −1 and −183°. Thawing was by immersion in water at controlled temperatures between 0 and 37°, the colder samples being first warmed up to −6°. Lipoprotein used for investigation of changes during storage in the frozen state was frozen and thawed under conditions found to cause minimal damage, i.e. by rapid freezing at low temperature and rapid thawing at 37°. The frozen material was held in closed vessels over ice to prevent desiccation.

Drying and storage in the dried state. For freeze-drying, the lipovitellin suspension (approx. 10% solids) was rapidly frozen in shallow layers in thin metal trays by immersion in solid CO_2-ether at −60 to −70°. The trays were then transferred to a refrigerated freeze-drying cabinet in a room at 0°, and the apparatus rapidly evacuated. Brine at −25 to −30° circulating through the cooling column of the drier carried away the heat of hydration of the P_2O_5 which, in trays well separated from the lipoprotein, absorbed the water. After 40 hr. the dried material (moisture content approximately 4·5%) was removed, thoroughly mixed in a blender at 0°, and sampled for dry weight determination (vacuum oven at 70°).

For storage at constant relative humidity (R.H.) samples of the dried lipoprotein (approx. 0·8 g.) were held at 37° in shallow aluminium dishes in evacuated vessels over P_2O_5 (0% R.H.) and over solutions of H_2SO_4 producing known water vapour pressures. In earlier experiments a preliminary equilibration with the storage atmosphere was carried out at a lower temperature, but the finely divided product of freeze-drying reached equilibrium very rapidly *in vacuo* at 37° and this precaution was subsequently omitted.

Table 1. *Effect of ether on the dispersibility of lipovitellin*

(Preparation 18, in NaCl solutions, w/v.)

	N in solution after 16 hr. at 3° (as % total N)					
pH	1% NaCl	1% NaCl-ether	5% NaCl	5% NaCl-ether	10% NaCl	10% NaCl-ether
5·0	1	1	10	12	11	13
5·5	1·5	1·5	26	34	33	36
6·0	2	2	46	69	56	77
7·0	6	6	51	78	65	88

RESULTS

Some properties of lipovitellin

Solubility. Saturation with ether (0·8 ml./10 ml. solution) considerably increased the dispersing power of aqueous sodium chloride solutions (Table 1). This amount of ether was therefore used in the routine determination of solubility.

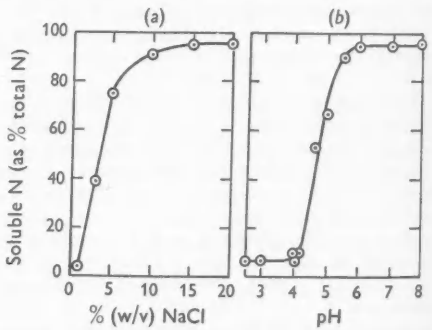

Fig. 1. Effect on the solubility of lipovitellin (preparations 18, 23, 28) of (*a*) NaCl concn. at pH 6·8 and (*b*) of pH at 15% (w/v) NaCl. Temp. 0°; total concn. of lipoprotein 2% (w/v); 0·8 ml. ether present/10·6 ml. solution.

The effects of varying ionic strength at constant pH, and of varying pH at constant ionic strength are shown in Fig. 1. A concentration of 15% (w/v)

sodium chloride and a pH of 6·0 were usually employed in comparing the solubilities of treated samples.

Extractability of the lipid. Fresh lipovitellin suspension at pH 6·8, when dispersed by the addition of sodium chloride and extracted with ether, as described above, yielded lipid to the solvent at a slow and linear rate of approximately 3 % of the total lipid present per hour (Fig. 2*a*). After adjustment to pH 5·2 with hydrochloric acid and standing 24 hr. at 0° a much larger proportion of the lipid was extractable with ether, 20 % being removed in 30 min. and 25 % in 2 hr.

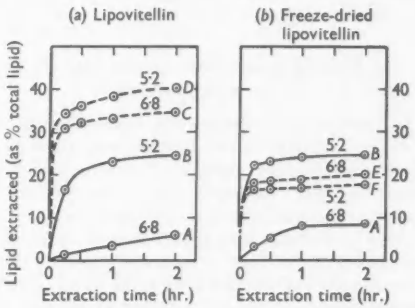

Fig. 2. Extractability with ether at 0° of lipid from (*a*) fresh (moist) and (*b*) freeze-dried lipovitellin (preparation 22). *A*, *B*, extraction from 10 % (w/v) NaCl containing 10 % (w/v) lipovitellin; *C*, *D*, extraction from 10 % (w/v) aqueous lipovitellin suspension. Anhydrous Na_2SO_4 (1·2 g./ml.) added; *E*, *F*, extraction of dry lipovitellin.

Addition of anhydrous sodium sulphate to the aqueous suspension-ether mixture, followed immediately by shaking, i.e. dehydration during extraction, resulted in a greatly increased liberation of free lipid, most of which was extracted in the first 15 min. After 2 hr. 35 % of the total lipid had been liberated at pH 6·8 and 40 % at pH 5·2 (Fig. 2*a*).

Effect of pH *and of sodium chloride on extractability of the lipid.* To investigate further the effect of pH on the extractability of the lipid, samples of lipovitellin suspension (approx. 10 % solids) were dispersed by the addition of sodium chloride (10 %, w/v) and the pH adjusted from the original value of 7·0 to the required figure by the slow addition of citric acid or dilute sodium hydroxide solution. After standing for 30 min. the suspensions were extracted by shaking with ether for 30 min. at 0°. The result (Fig. 3) showed that only a trace of free lipid was extractable by ether at pH values above 5·5, but that increasing amounts were extracted as the pH was reduced below this point. A sample of the suspension acidified to pH 5·2 with hydrochloric acid gave the same result as with citric acid, but holding the acidified suspension at 0° for 16 hr. before extraction increased the proportion of the lipid extracted from 7 to 13 %.

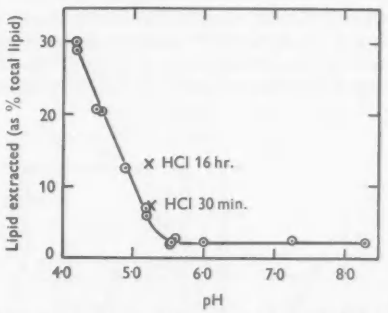

Fig. 3. Effect of pH on the extractability of the lipid of lipovitellin (preparation 28) from 10 % (w/v) NaCl with moist ether at 0°. pH adjusted with citric acid and extracted after 30 min. except where indicated.

Dialysed aqueous suspensions of lipovitellin at approximately neutral pH were resistant to extraction by ether, but the addition of very small quantities of sodium chloride caused the liberation of free lipid, although the amount fell again as the concentration of sodium chloride was further increased and the lipoprotein began to dissolve (Fig. 4). It was observed that the aqueous phase showed an increased tendency to disperse in the ether and to settle less readily after shaking at sodium chloride concentrations which gave the highest yields of free lipid.

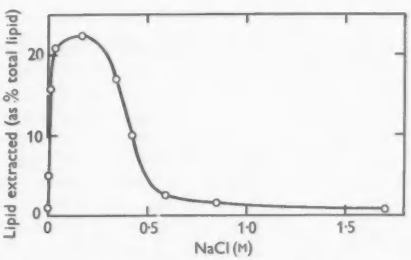

Fig. 4. Effect of NaCl on the extraction of lipid from aqueous lipovitellin (preparation 29) at pH 6·8 by shaking with moist ether (100 ml. ether/3 g. 10 % (w/v) suspension) for 15 min.

Isoelectric point. Since lipovitellin is practically insoluble in water and in dilute buffers, an approximate estimate of its isoelectric point was obtained by determination of the pH at which the addition of sodium chloride neither increased nor decreased the apparent hydrogen-ion concentration (Schmidt, 1945). For this purpose, a concentrated lipovitellin

suspension (approx. 10 % solids) was adjusted to the required initial pH value by the addition of dilute hydrochloric acid, and the effect of adding a small quantity (0·021 M) of sodium chloride measured. The results (Fig. 5*a*) indicated an isoelectric point in the region of pH 5·50. With another preparation of lipovitellin (no. 25) additions of 0·011, 0·021, 0·043 and 0·086 M-sodium chloride gave isoelectric points at pH's 5·59, 5·53, 5·56 and 5·55.

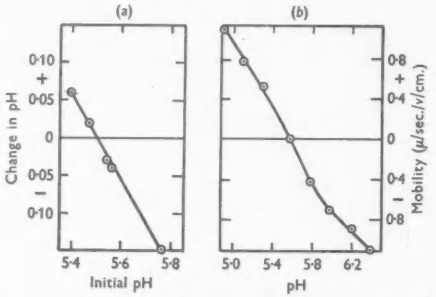

Fig. 5. The isoelectric point of lipovitellin (preparation 26). *a*, Changes in pH produced in lipovitellin suspensions by the addition of NaCl (0·021 M); *b*, electrophoretic mobility as a function of pH in 0·0033 M-phosphate-citrate buffers.

In an alternative procedure, the determination was carried out electrophoretically using the cell perfected by McQuillen (1950) for microscopic observation of the movement of bacterial cells in a reversible electric field. Mobilities were measured for a considerable number of particles of lipovitellin over a range of pH values in very dilute citrate-phosphate buffer. These values plotted against pH indicated zero mobility in the region of pH 5·57 (Fig. 5*b*).

Stability at 37°. Fresh lipovitellin suspension decomposed comparatively rapidly at 37° whether the criterion was loss of solubility or increase in ether-extractable lipid (Table 2). Increasing the total salt present from 0·9 % (w/v) sodium fluoride (used as a bacterial inhibitor) to 10 % (w/v) by the addition of sodium chloride had no very marked effect on the stability of the lipoprotein. The rate of liberation of lipid from all three preparations was somewhat greater than previously reported (Lea & Hawke, 1951), but the total lipid contents were also considerably higher.

Effect of freezing and thawing, and of storage in the frozen state

In preliminary experiments, more damage was found to result from the freezing and thawing of lipovitellin suspensions than from freeze-drying. Attention was therefore first directed to the thawing process. Table 3 shows that, after freezing at −65°, solubility was best preserved by thawing as rapidly as practicable in water at 37°. Under these conditions changes were small at pH 6·8, but were still large at pH 5·2 with the lipovitellin preparation used. When rapid thawing was standardized, the lipoprotein suffered less loss of solubility the lower the freezing temperature, i.e. the more rapid the freezing (Table 4).

The proportion of the total lipid extractable with ether after freezing and thawing appeared to be largely independent of the temperature of freezing,

Table 2. *Stability of fresh lipovitellin suspensions and of remoistened, freeze-dried lipovitellin at* 37°

Preparation no.	pH	Composition of solvent*	Days at 37°: Lipovitellin 0	Lipovitellin 2	Lipovitellin 5	Freeze-dried lipovitellin 0	Freeze-dried lipovitellin 1	Freeze-dried lipovitellin 2	Freeze-dried lipovitellin 5
			Percentage of total N soluble						
22	6·8	*A*	93	52	52	87	—	47	39
22	6·8	*B*	93	50	50	87	—	39	37
23	6·8	*A*	97	52	—	—	—	—	—
20	6·8	*A*	—	—	—	94	62	53	40
22	5·2	*A*	89	38	38	60	—	17	12
22	5·2	*B*	89	28	28	60	—	12	10
23	5·2	*A*	87	39	—	—	—	—	—
20	5·2	*A*	—	—	—	94	30	19	—
			Percentage of total lipid free†						
22	6·8	*A*	1·5	11	14	3	—	17	26
22	6·8	*B*	1·5	10	13	3	—	10	16
22	5·2	*A*	16	23	32	22	—	25	33
22	5·2	*B*	15	19	27	22	—	23	26
23	5·2	*A*	15	20	—	—	—	—	—

* *A*, 0·9 % (w/v) NaF; *B*, 0·9 % (w/v) NaF + 9·1 % (w/v) NaCl.
† Wet extraction method.

and averaged 2% at pH 6·8 and nearly 40% at pH 5·2, part of the latter being attributable to acidification rather than to freezing. The amount of free lipid produced by freezing lipovitellin at pH 6·8 varied appreciably in different preparations (e.g. 2, 8, 14 and 9% in preparations 21, 23, 24 and 25 respectively), although altering the temperature, and hence the rate of freezing still showed no marked effect.

Table 3. *Effect of speed of thawing (after freezing rapidly at −65°) on the solubility of lipovitellin (preparation 22)*

Thawing temperature (°)	Thawing time (min.)	Solubility (%)* after freezing and thawing at pH 6·8	pH 5·2
Not frozen	—	92·6	88·8
37	0·7	88·3	53·7
5·4	3·7	84·7	43·9
1·0	12	86·2	47·6
0·0	35	71·8	37·1

* In 15% (w/v) NaCl—ether at pH 6.

Table 4. *Effect of freezing at various temperatures on lipovitellin*

(Preparation 21; thawing at 37°.)

pH	Temp. of freezing (°)	Solubility (%)	Percentage of total lipid free
6·8	−3	55	2
6·8	−7	55	2
6·8	−20	66	2
6·8	−65	63	—
6·8	−183	74	1
5·2	−3	17	40
5·2	−7	19	38
5·2	−20	21	36
5·2	−65	36	36
5·2	−183	40	42

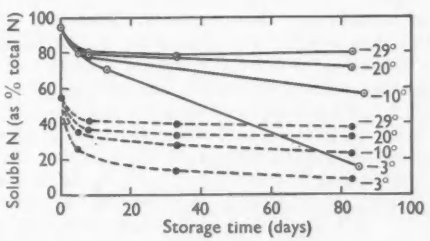

Fig. 6. Effect of storage temperature on the development of insolubility in frozen lipovitellin at pH 6·8 (⊙) and pH 5·2 (●). Preparation 23.

To isolate the effects of storage in the frozen state a lipovitellin suspension was frozen at −65°, stored at various constant temperatures below 0° and thawed rapidly in water at 37°. The results (Fig. 6) show that solubility decreased rapidly at first at both pH's and at all four storage temperatures, and thereafter decreased more slowly at rates which varied with the temperature from practically zero at −29° to a rate at −3° which produced almost complete insolubility within 3 months. At pH 6·8, very little free lipid was split off during 3 months at −29° and only slightly more at −20°. The liberation of lipid however was considerable at −10° and particularly at −3°. At all four temperatures, most of this change occurred during the first 10 days of storage (Fig. 7). At −3° the appearance of the samples changed from white to translucent, presumably owing to the growth of the larger ice crystals at the expense of the small.

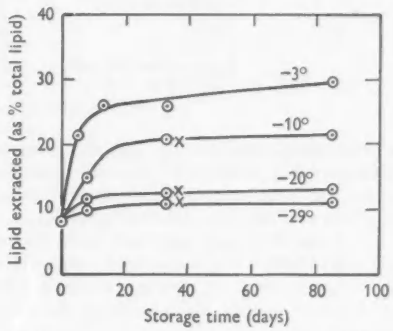

Fig. 7. Effect of storage temperature on the liberation of lipid from lipovitellin suspension (pH 6·8) frozen at −65°. (Thawed at 37°; ⊙, preparation 23; ×, preparation 25.)

Effect of freeze-drying

Solubility. Freeze-drying of lipovitellin suspensions by the described procedure had a variable effect on the dispersibility of the lipoprotein in 15% (w/v) sodium chloride-ether at pH 6 (Table 5), some preparations apparently being more susceptible to damage than others. Solubility in all cases was higher when drying was at pH 6·8 than at pH 5·2, and the dried product in both cases could be held at −20° for some weeks with little further change.

Extractability of the lipid. A considerable proportion of the lipid of freeze-dried lipovitellin was extractable with ether. Four preparations of the lipoprotein freeze-dried at pH 6·8 yielded 24, 24, 30 and 20% (average 24·5%) of extractable lipid. At pH 5·2 the yields were slightly lower, namely 22, 18, 26 and 18% (average 21%). Extraction was almost complete after 15 min. (Fig. 2*b*). If, however, the material dried at pH 6·8 was re-moistened with water before extraction, very much smaller quantities of lipid passed into the ether, and the proportion of the total lipid removed in 2 hr. (8–9%) was only slightly greater than from undried lipo-

Table 5. *Effect of freeze-drying on the solubility of lipovitellin*

pH of suspension	Treatment	Preparation no. 20	22	23*	24*
		Solubility (%) in 15% (w/v) NaCl-ether at pH 6			
6·8	Fresh	94·7	92·6	96·7	94·4
	Freeze-dried	94·2	86·8	76·5	70·3
	Freeze-dried and held 13 days at −20°	94·1	—	—	—
5·2	Fresh	94·7	88·8	87·3	88·1
	Freeze-dried	94·0	60·2	55·5	56·5
	Freeze-dried and held 13 days at −20°	93·8	—	—	—

* Sucrose present during the preparation (cf. p. 105).

vitellin similarly treated (Fig. 2*b*). With another preparation the difference between the fresh material and that re-moistened after drying at pH 6·8 was still smaller. On the other hand, extraction of re-wetted material which had been dried at pH 5·2 yielded even more lipid than did dry extraction, the limiting amount reached (25%) being the same as that liberated from the undried lipoprotein.

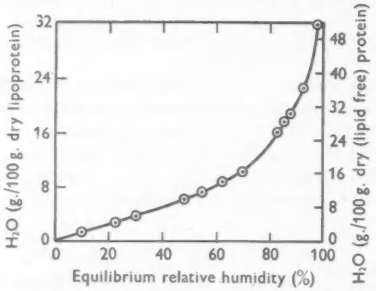

Fig. 8. The vapour pressure isotherm of freeze-dried lipovitellin (preparation 20) at 37°. Lipid content 38·7% of dry weight.

Stability after re-moistening. A comparison of the behaviour on keeping at 37° of lipovitellin and of the same material after freeze-drying and re-moistening to the original water content is given in Table 2. Although some decomposition frequently resulted from the drying process itself, little if any increase in susceptibility to further decomposition on storage of the moist material at 37° was apparent.

The vapour-pressure isotherm of freeze-dried lipovitellin. The relationship between the equilibrium relative humidity and the moisture content of freeze-dried lipovitellin as determined by drying to constant weight *in vacuo* at 70° is given in Fig. 8. The isotherms at pH's 6·8 and 5·2 were indistinguishable. The storage experiments with dried material were carried out at constant relative humidities.

Effect of storage in the dried state

Solubility. Freeze-dried lipovitellin in equilibrium with atmospheres of high relative humidity, i.e. at high moisture contents, became insoluble extremely rapidly, much more rapidly, in fact, than when suspended in water or dispersed in sodium chloride solution (Table 2). In a completely dry atmosphere the rate of change was very slow, while atmospheres of intermediate composition produced intermediate rates of change (Fig. 9). In all cases, insolubility developed considerably more rapidly in the material adjusted to pH 5·2 before drying than in that adjusted to pH 6·8. A perceptible lag or induction period noticeable with certain of the samples which became insoluble at intermediate rates was not detectable under conditions producing more rapid deterioration.

Extractability of the lipid. Fig. 10 shows that, qualitatively, the effects of storage in moist and dry atmospheres on the liberation of free lipid (as estimated by extraction of the 'dry' lipoprotein) were similar to the effects on solubility, the changes being very rapid at high humidites and very slow at low humidities. Quantitatively, however, the parallelism was far from complete. For example, the liberation of free lipid seemed always to be complete within 1 day at 37°, and samples held at 10% R.H. for as long as 45 days still gave the same free lipid values as at 1 day, i.e. 31·5 and 28% respectively at pH's 6·8 and 5·2. The solubility data at pH 6·8 and 70 or 85% R.H., and at pH 5·2 and 30% R.H., on the other hand, showed an 'induction period' of 1 day during which very little loss of solubility occurred, although thereafter the change was rapid and speedily approached a limiting value. Furthermore, at intermediate R.H. values, such as 70% R.H. at pH 6·8 and 70, 55 and 30% R.H. at pH 5·2, the loss of solubility during storage was relatively very much more marked than the liberation of lipid (Figs. 9 and 10).

The free lipid estimations in the storage experiments were normally carried out by extraction of

the solid ('dry') lipoprotein with ether, but extraction by the 'wet' method, i.e. after redispersion in sodium chloride solution, showed generally similar changes in an experiment in which the two methods were tested against one another on a decrease in rate at high humidities with any certainty. The cause of the darkening on storage has not yet been ascertained: it may be due to autoxidation of the lipid, or to a 'browning' reaction of the amino-aldehyde type, or to both.

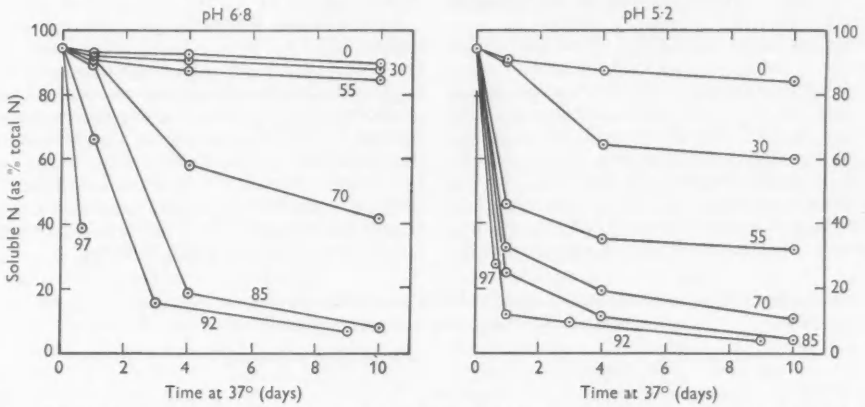

Fig. 9. Effect of variation in moisture content (equilibrium R.H. indicated) on the development of insolubility in freeze-dried lipovitellin (preparation 20).

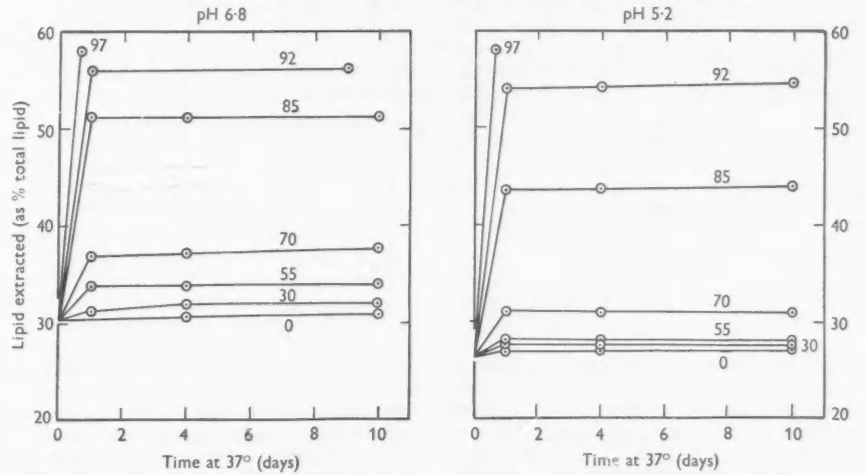

Fig. 10. Effect of variation in moisture content (equilibrium R.H. indicated) on the liberation of free lipid (dry-extraction method) from lipovitellin (preparation 20).

different (and apparently more stable) specimen of freeze-dried lipovitellin which had been stored at 85% R.H. (Table 6).

Colour. The slightly 'off-white' colour of freshly freeze-dried lipovitellin darkened on storage, the change being greatly accelerated by an acid pH and a high relative humidity (Table 7). The rate of discoloration appeared to be maximal at about 85% R.H., but the data are too few to establish the

DISCUSSION

A troublesome feature of this work on the freezing and drying of lipovitellin has been a certain lack of exact reproducibility in the behaviour of preparations made in apparently the same way when submitted to apparently similar freezing and drying treatments. It is not known whether these differences derived from the eggs, which were collected

from various sources and at various seasons, or from chance variations in technique. Despite this limitation, the results have some interest both from the theoretical viewpoint of the properties and structure of lipoproteins and from the more practical one of the behaviour of these substances in biological materials preserved by freezing or drying.

State of the lipid in lipovitellin. Fresh lipovitellin dispersed in 10 % (w/v) sodium chloride solution gave up lipid to ether only very slowly at pH values above 5·5, but up to one-third of the lipid could be extracted rapidly from more acid salt solutions (Fig. 3) in which the lipoprotein was much less soluble. A similar proportion of the lipid could also be obtained at neutral pH from suspensions of lipovitellin in sodium chloride solutions too weak to disperse it. It seems reasonable to suppose that the extraction, and even more (30–35 %) if the moist lipovitellin were dried by sodium sulphate during extraction (Fig. 2), it would appear that water has some function in stabilizing the union of the more loosely held portion of the lipid with the remainder of the complex.

To this extent, therefore, lipovitellin seems to be decomposed by drying. On the other hand, when lipovitellin, which has been freeze-dried at pH 6·8, is re-wetted, it is found to retain most of its solubility and to have recovered most of its capacity for withholding its lipid from ether: at this pH, at least, decomposition produced by drying seems to be largely reversible. At pH 5·2 the loss of solubility on freezing or drying was usually much more marked than at pH 6·8, and the lipoprotein lacked resistance to ether even before freezing or drying.

Table 6. *Liberation of free lipid from lipovitellin (preparation* 24*) stored at* 37° *and* 85 % R.H., *as measured by 'dry' and 'wet' extraction methods*

	Free lipid (as % total lipid)					
	pH 6·8			pH 5·2		
Stored at 37° (days)	0	1	6	0	1	6
'Dry' extraction	28	38	39	25	33	34
'Wet' extraction	4	20	25	26	34	35

Table 7. *Effect of* pH *and moisture content on the discoloration of freeze-dried lipovitellin at* 37° * (*preparation* 20)

	Time stored	Equilibrium relative humidity (%)					
pH	(days)	0	30	55	70	85	92
6·8	1	—	1·5	1·4	1·4	1·4	1·5
	4	1·4	1·4	—	1·3	1·4	1·5
	10	1·6	1·5	1·4	1·5	1·8	1·8
5·2	1	1·4	—	1·4	1·8	1·8	1·7
	4	1·6	1·7	1·6	2·0	3·5	2·5
	10	—	1·6	1·7	—	4·5	3·9

* Initial colour, 1·4 $R + Y$ Lovibond units.

lipids of lipovitellin in 'solution' are inaccessible to ether through the whole lipid molecules, or at least their lipophilic portions, being orientated towards the interior of the lipoprotein molecule, away from the water surface. The extraction of a considerable amount of lipid from the moist particle in suspension in the presence of insufficient sodium chloride to disperse it, but not from suspension in pure water (Fig. 4), is difficult to account for, and may be due to some mechanical factor such as more intimate contact between the aqueous and ether phases as indicated by the slower separation of the dilute salt solution from the ether after shaking. There was a marked similarity between the amounts of lipid liberated under these conditions and by drying.

Since 15–20 % of the lipid was extractable within 10 min. if the lipoprotein were freeze-dried before

Under most of the conditions investigated the amount of free lipid liberated tended to rise towards some limiting value which could be anything from 10 to 50 % or even more, a circumstance which seemed to indicate a far from uniform strength of binding between different fractions of the lipid and the protein. Tayeau (1944) believes that neutral lipid is mainly bound to the phospholipins, and that phospholipins form the link between neutral lipid and protein. With such a structure, it might be possible to break selectively the protein-lipid and lipid-lipid associations in the lipoprotein, and Delsal (1949) has already found that 'delipidation' by anhydrous ether at − 70° according to the method of McFarlane (1942) appears to extract only cholesterol from blood serum, the phospholipin-protein 'cénapses' remaining intact. An investigation of the

chemical nature of the lipid of lipovitellin and of the fractions removed by various physical and chemical treatments is now being undertaken.

Effect of freezing. Freezing and thawing tended to destroy the solubility of lipovitellin, the effect being greater at pH 5·2 than at pH 6·8. The damage could be reduced by freezing rapidly at low temperature and by thawing rapidly (Table 3), and there was no sign of increasing loss of solubility as more water was frozen out by lower freezing temperatures—in fact, the reverse tended to be the case (Table 4). When stored in the frozen state the lipoprotein decomposed at an appreciable rate even at low temperatures, and the difference between -20 and $-29°$ could readily be detected after 2 or 3 months (Figs. 6 and 7). Obviously, the lipoproteins are among the constituents of biological materials most susceptible to decomposition during storage.

In freezing mammalian and fish muscle Moran (1935) and Reay (1933) have drawn attention to the rapid denaturation of the protein which occurs at temperatures just below the freezing point of the muscle, and particularly in the region of -2 to $-4°$, and Finn (1932) has attributed these results to the increased concentrations of muscle salts and hydrogen ions which develop in the liquid phase of the material at these 'critical' temperatures when a large proportion of the water freezes out. These natural muscle systems, however, are not comparable with the dialysed protein suspensions used in the present work, in which the concentration of salts is very low. Comparison with the experiments of Moran (1925), who was able to freeze egg yolk rapidly in liquid air and thaw it rapidly in warm mercury without the serious loss of fluidity which occurred if freezing or thawing were slow, is similarly complicated by the presence of salts in the whole yolk. Our results on the effect of pH on the stability of lipovitellin suggest that damage could easily be produced by a depression of the natural pH of the yolk (6·1–6·2) such as could be produced by concentration of the salts during freezing.

Effect of drying. Lipovitellin could usually be freeze-dried at pH 6·8 with but little decomposition, and one sample was dried even at pH 5·2 without loss of solubility, although deterioration at this latter pH was usually marked. Lipovitellin therefore appears to be more stable than the lipoproteins of human plasma (Gurd, Oncley, Edsall & Cohn, 1949; Oncley, Gurd & Melin, 1950).

The results of storage in the dried state were particularly interesting in showing a high degree of stability at very low moisture contents and a rapid rate of deterioration at high moisture contents, with intermediate values at intermediate moisture contents (Figs. 9 and 10). Deterioration in the moist solid was, in fact, much more rapid than in the fully wetted state or in 'solution'. This result, while in keeping with the non-harmful effect of freezing at very low temperatures, is in apparent conflict with the theory that water forms an essential part of the lipoprotein complex. It could perhaps be conceded that, in a union split by drying, the components might be unable to undergo further change under very dry conditions and would combine again on rehydration. The failure of ether extraction to remove increased amounts of lipid from the dried material after storage at low humidities, whilst readily doing so from dried material after storage at high humidities (Fig. 10), however, could more easily be explained by a theory of increasing liberation of lipid due to progressively greater denaturation at increasing moisture contents of the protein with which it had been combined. On the other hand, loss of solubility and liberation of free lipid do not always bear the same relationship to one another (cf. p. 110), and the assumption would be necessary that in some cases an appreciable interval elapses between the denaturation which liberates the lipid and the aggregation which results in the loss of solubility. Even this explanation does not cover the few cases already referred to (p. 110) where a marked loss of solubility accompanied a relatively minor liberation of lipid, i.e. where denaturation appeared to precede splitting. The precise nature of the connexion between loss of solubility and liberation of free lipid has not, therefore, been established.

From the viewpoint of practical dehydration and storage the desirability of drying to low ultimate moisture contents and, during drying, of passing rapidly and at low temperature through the range of critical moisture contents where deterioration is rapid can readily be seen from Figs. 9 and 10.

SUMMARY

1. The effects of freezing and drying lipovitellin and of storage in the frozen and dried states have been investigated.

2. Decomposition of the lipoprotein was minimized by rapid freezing and thawing, and was not increased by very low freezing temperatures. Further changes occurred during storage in the frozen state at temperatures down to $-29°$, and at $-3°$ the lipoprotein became completely insoluble.

3. Lipovitellin could be freeze-dried at approximately neutral pH with but little decomposition, and was then comparatively stable at low storage temperatures, or even at 37° if very dry. In equilibrium with atmospheres of high relative humidity the moist solid decomposed much more rapidly than either the dry solid or the fully moistened or dispersed lipoprotein.

4. About one-quarter of the lipid of freshly freeze-dried lipovitellin was readily extractable with ether, but on moistening (at neutral, but not at acid,

pH) most of this lipid again became extractable only with difficulty.

5. The parallelism between the two criteria of decomposition used, namely dispersibility in sodium chloride solution-ether and extractability of the lipid with ether, was not complete.

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REFERENCES

Bate-Smith, E. C. (1935). *Report of the Food Investigation Board*, p. 35. London: H.M. Stationery Office.
Brooks, J. & Hawthorne, J. R. (1944). *J. Soc. chem. Ind.* **63**, 310.
Chargaff, E. (1942). *J. biol. Chem.* **142**, 491.
Delsal, J. L. (1949). *Bull. Soc. Chim. biol.* **31**, 122.
Dervichian, D. G. (1949). *Lipoproteins.* Discussions of the Faraday Society, London, no. 6, p. 13.
Fevold, H. L. & Lausten, A. (1946). *Arch. Biochem.* **11**, 1.
Finn, D. B. (1932). *Proc. roy. Soc.* B, **111**, 396.
Gurd, F. R. N., Oncley, J. L., Edsall, J. T. & Cohn, E. J. (1949). *Lipoproteins*, Discussions of the Faraday Society London, no. 6, p. 70.
Lea, C. H., Hannan, R. S. & Rhodes, D. N. (1951). *Biochim. biophys. Act. Amst.* **7**, 366.
Lea, C. H. & Hawke, J. C. (1951). *Biochem. J.* **50**, 67.
McFarlane, A. S. (1942). *Nature, Lond.*, **149**, 439.
McQuillen, K. (1950). Ph.D. Thesis, University of Cambridge.
Moran, T. (1925). *Proc. roy. Soc.* B, **98**, 436.
Moran, T. (1935). *J. Soc. chem. Ind.* **54**, 149T.
Oncley, J. L., Gurd, F. R. N. & Melin, M. (1950). *J. Amer. chem. Soc.* **72**, 458.
Reay, G. A (1933). *J. Soc. chem. Ind.* **52**, 265T.
Schmidt, C. L. A. (1945). *The Chemistry of Amino Acids and Proteins*, 2nd ed. p. 1213. Springfield: Charles C. Thomas.
Tayeau, F. (1944). *Bull. Soc. Chim. biol.* **42**, 715.

The Effects and the Fate of Malononitrile and Related Compounds in Animal Tissues

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It is generally accepted that the toxicity of aliphatic nitriles in the animal body is mainly due to a gradual release of cyanide. The evidence on which this view is based consists (*a*) in the similarity of the symptoms of intoxication to those of cyanide poisoning, with due consideration of the different rates at which cyanide is liberated from different nitriles, (*b*) in the antidotal effect of thiosulphate, and (*c*) in the increased excretion of thiocyanate (cf. Hunt, 1923). The second and third facts are to be attributed to the action of the enzyme rhodanese which catalyses the reaction (Lang, 1933):

$$HCN + Na_2S_2O_3 = HSCN + Na_2SO_3.$$

The formation of cyanide from nitriles has, however, not yet been demonstrated *in vitro*. Lang (1933) investigated the formation of SCN^- from acetonitrile and propionitrile by minced tissues with negative results. Warburg (1911; Warburg & Wiesel, 1912), who found an inhibition of the respiration of avian erythrocytes and of the fermentation of yeast cells by high concentrations of mononitriles, came to the conclusion that this was an unspecific 'narcotic' effect.

Recently, Hydén & Hartelius (1948) described an effect of malononitrile which they could not reproduce with cyanide. Using the microspectrographic method they found, in experiments on rabbits, an increase in the concentration of cytoplasmic protein and nucleoprotein of ganglion cells 1–48 hr. after an injection of malononitrile. Their observations are at variance with the results of Hicks (1950), Liu (1951) and Reale & D'Argenio (1951) using histological techniques, and with those of Samuels, Boyarsky, Gerard, Libet & Brust (1951) using chemical methods. Nevertheless, the general importance of the claims of Hydén & Hartelius and the particular interest arising from their recommendation of malononitrile therapy in mental diseases suggested an investigation into the effects of malononitrile and other nitriles on the metabolism of tissue slices, and into the correlation of any such effects with the release of cyanide. As Heymans & Masoin (1897) have shown, the toxic action of malononitrile is fast; it seemed therefore well suited for short *in vitro* experiments.

Another class of compounds from which cyanide is gradually liberated, in the body as well as in

presence of tissue preparations, is the group of alkyl thiocyanates, especially the lower homologues of the series (Oettingen, Hueper & Deichmann-Gruebler, 1936). Methyl and ethyl thiocyanates were therefore included amongst the substances studied.

EXPERIMENTAL

Methods

Measurement of tissue metabolism. Tissue slices were used for metabolism experiments. They were cut free-hand with a safety-razor blade moistened with Ringer solution, pooled and kept on ice. The manometer vessels were also kept on ice until they were attached to the manometers. All results were calculated on the basis of the initial dry weight: the slices, freed from adhering moisture by filter paper, were weighed on the torsion balance and the dry weight calculated from the ratio determined on a separate sample which was removed from the pool simultaneously with the other slices and dried at 100°. Owing to the swelling of the slices in Ringer solution the ratio for brain slices varied between 5·5 and 7·0.

When respiration alone was measured, 'medium III', a saline low in phosphate and bicarbonate, but including organic salts (Krebs, 1950), was used. In other experiments glycolysis was determined by chemical estimation of lactic acid while respiration was recorded manometrically. The medium used for these experiments differed from medium III only by the omission of the three organic salts and their replacement by isotonic NaCl; it will be referred to as 'medium III, modified'. Respiration was followed in conical Warburg flasks containing NaOH in the inner well and O_2 in the gas space. When lactic acid was determined chemically, 0·1 vol. of 30% (w/v) trichloroacetic acid was added from the side bulb at suitable intervals. The first vessel was acidified at the time of starting the readings and served as initial value. Lactic acid estimations were usually carried out on 0·3 ml. of filtrate by the method of Barker & Summerson (1941) as modified by Umbreit, Burris & Stauffer (1945).

In another series of experiments respiration and glycolysis were determined simultaneously by the two-vessel method of Warburg (1924) in a bicarbonate-glucose medium (Krebs & Henseleit, 1932) with O_2–5% CO_2 as gas phase.

Respiratory quotients (R.Q.) were determined in bicarbonate medium by the method of Dickens & Šimer (1931).

Anaerobic glycolysis was measured manometrically in bicarbonate medium in an atmosphere of N_2–5% CO_2. The values for the aerobic glycolysis recorded in Figs. 1 and 2 have been expressed as a percentage of the anaerobic glycolysis. In these experiments slices were incubated in medium III, modified, in N_2, for 30 min. and lactic acid was determined by chemical estimation. This was done in order to measure aerobic and anaerobic glycolysis under identical conditions.

For the investigation of the effects of cyanide, Dickens-Šimer vessels (bicarbonate type) were used. The outside circular trough contained 1 ml. of alkaline KCN solution (Umbreit *et al.* 1945). Medium and tissue were introduced into the inner chamber and, after the vessels had been gassed, they were quickly removed from the manometers for the addition of the requisite amount of neutral KCN to the medium. All tissue slice experiments were done at 37°.

Tissue homogenates and extracts. Tissues were homogenized in an all-glass homogenizer (Potter & Elvehjem, 1936) with ice-cooled, glass-distilled water, usually in the ratio 1:10. For the preparation of extracts, the homogenates were centrifuged for 10 min. in an angle centrifuge at 3500 rev./min. and the supernatant was used. When experiments were carried out with boiled preparations, the required volume was pipetted into the empty reaction flask, which was stoppered and put in a boiling-water bath for 10 min. After cooling, the other constituents were added.

Homogenates and extracts of liver were prepared from rats which had been starved for 24 hr. before death.

Estimation of cyanide. The method of Epstein (1947), as modified by Boxer & Rickards (1951), has been adapted for our purpose. Aeration was carried out in 4–5 trains connected in series to a water-jet pump. Before entering the train the air stream was passed through 2N-NaOH; it was then led through the sample containing up to 2 μmoles CN^-, through a trap containing 20 ml. acid ceric sulphate (0·5N-solution in N-H_2SO_4) and finally through two collection tubes each containing 2 ml. 0·1N-NaOH. Entrainment of spray was prevented by suitable traps. Fresh ceric sulphate solution was used for each run and the train was aerated for about 1 hr. before the addition of the cyanide and NaOH solutions. Thereafter aeration was continued, at a moderate rate, for 90 min. The cyanide samples usually contained 5% trichloroacetic acid and required no further acidification.

Various modifications were also applied to the colorimetric procedure. The following stock solutions were used: (1) chloramine T, 1%; (2) 3-methyl-1-phenyl-5-pyrazolone (recrystallized three times), 0·4% solution in pyridine; (3) bis(3-methyl-1-phenyl-5-pyrazolone), 0·1% solution in pyridine. Solutions 2 and 3 were stored in a freezing compartment. Immediately before the analysis, 1 ml. of solution 1 was mixed with 3 ml. M-NaH_2PO_4 (solution 4; cf. Boxer & Rickards, 1951). Further, a pyrazolone-pyridine reagent (solution 5) was prepared by mixing 1 part of solution 2, 1 part of solution 3 and 6 parts of water. A 1 ml. sample containing not more than 0·3 μmole CN^- was cooled in ice, 0·2 ml. of solution 4 were added and, after thorough mixing, the tube was left at 0° for 2 min. Then 6 ml. of solution 5 were added and the mixture was kept at 30° for 30 min. Ilford spectrum filter no. 608 was used for the photoelectric measurement.

Malononitrile, methyl and ethyl thiocyanate did not interfere with the recovery of CN^-; they did, however, slightly increase the reagent blank, probably owing to the presence of traces of CN^- in the solutions.

Estimation of thiocyanate. 2 ml. of ferric nitrate solution (Cosby & Sumner, 1945) were added to 8 ml. of solution containing 0·2–4·0 μmole SCN^-. The red colour was determined photoelectrically after 15 min. with Ilford spectrum filter no. 601. A calibration curve was constructed with solutions containing the same concentration of buffer, thiosulphate and trichloroacetic acid as the unknown sample, and a standard solution and reagent blank were included with each series of estimations. Neither malononitrile nor the alkyl thiocyanates interfered with the reaction.

For deproteinization, the sample was mixed with an equal amount of 10% (w/v) trichloroacetic acid containing 0·14% formaldehyde (Rosenthal, Rogers, Vars & Ferguson, 1950).

Estimation of cozymase. The deproteinized filtrates were freed from trichloroacetic acid by three extractions with

peroxide-free ether (about 5 ml. each time). Ether was removed from the aqueous solution *in vacuo* at about 30°. The pH was adjusted to 6·4 and the volume made up to about ten times the weight of fresh tissue used. Cozymase concentration in the extract was determined by the apozymase test at 20°, as described by Schlenk & Schlenk (1947).

Cozymase, prepared by the method of LePage (1947), was standardized spectrophotometrically according to Kornberg (1950) and had a purity of 48·5 %.

Materials

Dimethylmalononitrile. Dimethylcyanacetamide was prepared from cyanacetamide (Corson, Scott & Vose, 1932) according to Errera (1896), and was dehydrated with PCl_5 as described for the preparation of malononitrile (Corson, Scott & Vose, 1943). Owing to the tendency of dimethylmalononitrile to sublime, the distillate was collected at −20° and the solidified product was repeatedly washed with ice-cold water and dried over P_2O_5 at atmospheric pressure; m.p. 30–32°.

All other substances were commercial products which were used without further purification. Malononitrile, redistilled *in vacuo* immediately before use, gave results which in no way differed from those obtained with the preparation before distillation. The crystals of malononitrile (m.p. 32°) were protected from light and stored at 2°. We did not notice any signs of decomposition under these conditions for many months.

RESULTS

The effects of nitriles and related compounds on tissue metabolism

The respiration of brain, kidney and liver slices is strongly inhibited by 0·01 M-malononitrile (Tables 1 and 2). The inhibition is progressive with time and is practically complete at the end of the second hour. In a bicarbonate medium, the respiration of brain and kidney slices is not significantly inhibited by 0·01 M-malononitrile during the first hour of the experiment, but in the second hour the effect is as strong as in phosphate medium. At the same time the aerobic glycolysis of brain slices is increased; it reached, in some experiments, 80–90 % of the level of the anaerobic glycolysis. Malononitrile, in

Table 1. *Effect of malononitrile on the respiration of tissue slices*

(Media: III = medium III (Krebs, 1950); III mod. = medium III, modified (cf. Methods section, p. 115); Additions: MN = malononitrile (0·01 M); 0 = none. Q_{O_2} values are in μl./mg. dry wt./hr.)

No.	Species	Tissue	Medium	Addition	Q_{O_2} 1st hr.	Q_{O_2} 2nd hr.	Inhibition (%) 1st hr.	Inhibition (%) 2nd hr.
1	Rabbit	Brain	III	0	−12·7	−10·1	—	—
				MN	−8·3	−4·5	35	55
2	Rat	Brain	III	0	−15·6	—	—	—
				MN	−10·3	—	34	—
3	Rat	Kidney	III	0	−27·0	−26·0	—	—
				MN	−20·0	−12·0	26	54
4	Rat	Kidney	III	0	−29·0	−24·0	—	—
				$Na_2S_2O_3$*	−27·0	−21·0	7	12
				MN	−19·0	−5·7	35	76
				MN + $Na_2S_2O_3$*	−23·0	−4·0	15‡	81‡
5	Rat	Liver	III	0	−11·6	−11·9	—	—
				MN	−6·8	−1·8	41	85
6	Rat	Liver	III	0	−11·4	−10·4	—	—
				$Na_2S_2O_3$*	−10·1	−8·8	11	15
				MN	−6·4	−1·5	44	85
				MN + $Na_2S_2O_3$*	−7·6	−2·3	25‡	74‡
7	Rat	Kidney	Bicarbonate	0	−20·3	−16·8	—	—
				$Na_2S_2O_3$†	−22·2	−11·8	0	30
				MN + $Na_2S_2O_3$†	−7·9	−2·2	64‡	81‡
8	Rat	Liver	Bicarbonate	0	−13·2	−12·4	—	—
				$Na_2S_2O_3$†	−13·3	−12·9	0	0
				MN	−4·9	−1·5	63	88
				MN + $Na_2S_2O_3$†	−3·0	−2·3	77‡	82‡
9	Rat	Kidney	III mod.	0	−16·7	−16·2	—	—
				$Na_2S_2O_3$†	−12·3	−11·7	26	28
				MN	−8·8	−5·9	47	64
				MN + $Na_2S_2O_3$†	−10·7	−5·7	13‡	51‡
10	Rat	Liver	III mod.	0	−9·4	−9·2	—	—
				$Na_2S_2O_3$†	−8·4	−6·8	10	26
				MN	−4·8	−2·6	49	72
				MN + $Na_2S_2O_3$†	−7·2	−1·1	14‡	84‡

* 0·05 M. † 0·035 M. ‡ Calculated on control + $Na_2S_2O_3$.

0·01 M-concentration, had little effect on the anaerobic glycolysis during the first hour (Table 3), but during the second hour both aerobic and anaerobic glycolysis tended to decrease. With lower concentrations of malononitrile there was no falling-off of the aerobic glycolysis during the second hour.

the inhibition of respiration caused by malononitrile was often partly relieved, but in the second hour respiration was equally inhibited in the presence and in the absence of thiosulphate (excepting Exp. 6, Table 2). Thiosulphate did not prevent the rise of the aerobic (Table 2) or the inhibition of the

Table 2. *Effect of malononitrile, methyl and ethyl thiocyanates on respiration and aerobic glycolysis of brain slices*

(Experiments in bicarbonate medium were performed by Warburg's two-vessel method. Where phosphate medium (medium III, modified) was used, lactic acid was determined chemically. Inhibitor concentration 0·01 M.)

				Q_{O_2}		Inhibition (%)		$Q_G^{O_2}$	
No.	Species	Medium	Addition	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.
1	Rat	Bicarbonate	0	−9·1	−9·2	—	—	+1·5	−0·2
			Malononitrile	−11·5	−2·5	0	73	+10·9	+7·2
2	Rat	Phosphate	0	−9·1	−9·5	—	—	+0·7	+0·3
			Malononitrile	−6·2	−3·3	31	65	+5·6	+5·6
3	Rat	Bicarbonate	0	−11·2	—	—	—	+2·7	—
			Methyl thiocyanate	−13·2	—	0	—	+15·2	—
			Ethyl thiocyanate	−12·0	—	0	—	+12·3	—
4	Rat	Phosphate	0	−11·3	−10·2	—	—	+2·2	+1·4
			Methyl thiocyanate	−6·3	−4·0	44	61	+8·8	+6·1
			Ethyl thiocyanate	−6·7	−5·2	41	49	+7·3	+5·1
5	Rat	Phosphate	$Na_2S_2O_3$*	−8·5	−9·2	—	—	+2·5	+4·1
			Malononitrile + $Na_2S_2O_3$*	−6·9	−3·5	19	62	+7·5	+8·0
6	Guinea pig	Bicarbonate	0	−8·9	−7·2	—	—	+2·6	+1·8
			Malononitrile	−6·9	−3·2	22	56	+18·0	+9·9
			$Na_2S_2O_3$*	−7·6	−7·0	15	3	+0·9	+0·7
			Malononitrile + $Na_2S_2O_3$*	−6·9	−5·5	9†	21†	+14·9	+12·4

* 0·035 M. † Calculated on control + $Na_2S_2O_3$.

Similar effects were observed with 0·01 M-methyl and ethyl thiocyanates (Table 2), but not with any of the following substances (all in a concentration of 0·01 M): acetonitrile, propionitrile, butyronitrile,

Table 3. *Effects of malononitrile and of thiosulphate on the anaerobic glycolysis of guinea pig brain slices*

Addition	$Q_G^{N_2}$ 1st hr.	$Q_G^{N_2}$ 2nd hr.
0	24·8	24·8
Malononitrile (0·01 M)	19·6	11·5
$Na_2S_2O_3$ (0·035 M)	23·7	29·6
Malononitrile + $Na_2S_2O_3$	18·7	13·5

*iso*valeronitrile, capronitrile, *iso*capronitrile, acrylonitrile, succinonitrile, glutaronitrile, dimethylmalononitrile, ethyl cyanoacetate. A slight inhibition of respiration and increase of aerobic glycolysis was found in some cases, but the effects are of doubtful significance. The numerical results of these experiments are therefore omitted.

Thiosulphate, which has such a dramatic effect as an antidote to malononitrile poisoning *in vivo*, has only a comparatively slight action *in vitro* (Tables 1 and 2). In the first hour of the experiment anaerobic glycolysis (Table 3) produced by malononitrile in brain slices. Thiosulphate itself has a slight inhibitory effect on respiration and may somewhat increase the aerobic glycolysis of brain slices, especially in the second hour.

For comparison, the antagonism between cyanide and thiosulphate was investigated in experiments with tissue slices (Table 4). Although the effects of cyanide were not completely reversed by thiosulphate, they were clearly alleviated. The inhibition of respiration by cyanide, unlike that caused by malononitrile, is not progressive with time; this is probably the reason why the antagonistic effect of thiosulphate is maintained in the second hour in the experiments with cyanide, but not in those with malononitrile.

The determination of respiration and aerobic glycolysis of brain slices by the Dickens-Šimer method confirmed the effects of malononitrile found with other methods. The R.Q. was not affected (Table 5).

Concentration effects. Fig. 1 shows the effects of increasing concentrations of malononitrile on the respiration and aerobic glycolysis of rat-brain slices in phosphate medium during the first hour. It appears that concentrations which inhibit

respiration by only about 10% raise the level of aerobic glycolysis to about 50% of that of the anaerobic glycolysis. It is noteworthy that respiratory inhibitions of this order were observed with 100 times smaller than those of malononitrile (Fig. 2). Here, too, aerobic glycolysis is inhibited at high concentrations, possibly owing to cyanohydrin formation.

Table 4. *Effect of cyanide and thiosulphate on the metabolism of rat-tissue slices*

(Phosphate saline (medium III, modified); cyanide 10^{-4} M, thiosulphate 0·035 M.)

No.	Tissue	Addition	Q_{O_2} 1st hr.	Q_{O_2} 2nd hr.	Inhibition (%) 1st hr.	Inhibition (%) 2nd hr.	$Q_G^{O_2}$ 1st hr.
1	Kidney	0	−18·8	−16·2	—	—	—
		$Na_2S_2O_3$	−16·2	−16·2	14	0	—
		KCN	−8·3	−7·3	56	55	—
		KCN + $Na_2S_2O_3$	−15·0	−12·4	7·4	23	—
2	Kidney	0	−17·9	−17·2	—	—	—
		$Na_2S_2O_3$	−17·7	−16·7	1	3	—
		KCN	−7·9	−7·0	56	59	—
		KCN + $Na_2S_2O_3$	−15·5	−14·2	12	15	—
3	Liver	0	−8·3	−8·3	—	—	—
		$Na_2S_2O_3$	−8·9	−7·5	0	10	—
		KCN	−4·7	−4·6	43	45	—
		KCN + $Na_2S_2O_3$	−6·8	−5·3	23	29	—
4	Liver	0	−8·4	−7·9	—	—	—
		$Na_2S_2O_3$	−8·7	−6·8	0	14	—
		KCN	−4·8	−3·4	43	57	—
		KCN + $Na_2S_2O_3$	−6·7	−5·4	23	21	—
5	Brain	0	−9·7	—	—	—	2·1
		$Na_2S_2O_3$	−8·9	—	8	—	4·6
		KCN	−3·1	—	68	—	12·1
		KCN + $Na_2S_2O_3$	−5·0	—	44	—	6·3

several of the other nitriles tested without the effect on aerobic glycolysis, suggesting a specific mechanism for the effect of malononitrile in contrast to an unspecific, 'narcotic' effect of other nitriles.

At concentrations of malononitrile greater than 0·03 M, the curve of the aerobic glycolysis shows no further rise, but rapidly declines until, at a concentration of 0·1 M, aerobic glycolysis is completely inhibited. This may be due, at least in part, to a direct reaction of malononitrile with glucose indicated by the appearance of a yellow colour, but, in addition, the unspecific, 'narcotic' action of malononitrile may be expected to become manifest.

Table 5. *Respiratory quotient of guinea pig brain slices in bicarbonate medium (Dickens & Šimer, 1931)*

(Duration of experiment, 2 hr.)

No.	Addition	Q_{O_2}	$Q_G^{O_2}$	R.Q.
1	0	−8·3	+0·6	1·01
	Malononitrile (0·01 M)	−4·5	+12·0	1·08
2	Malononitrile (0·01 M)	−5·7	+8·6	0·96

The curves of respiratory inhibition and aerobic glycolysis are of a similar shape and show a similar relationship to each other when cyanide is used instead of malononitrile, except that the effects occur at concentrations of cyanide which are about

The effect of malononitrile injections on the concentration of lactic acid and cozymase in rat brain

Male white rats were injected subcutaneously with 14 mg./kg. of malononitrile in 0·35% aqueous solution. Though this is a near-lethal dose, none of the injected rats succumbed within 2 hr. The animals were dyspnoeic and cyanotic and were lying quietly in their cages. Convulsions were occasionally seen. In some rats the effects had largely worn off after 2 hr. A second injection, equal to the first, was given to three animals after 1 hr.

The rats were killed after 2 hr. by immersion in liquid oxygen. The brains were dissected while frozen and crushed in a cooled steel mortar (LePage, 1948). The frozen powder was transferred to tared weighing bottles containing 10% trichloroacetic acid; these were then re-weighed. A series of control values was obtained by analysing the brains of normal rats in the same way.

The results obtained after malononitrile injection are collected in Table 6. In the first five rats the values were within the normal variation. Though these animals showed no obvious difference in behaviour and appearance from the rest, the effects of malononitrile had presumably largely subsided when they were killed. The other ten experiments show a significant increase in lactic

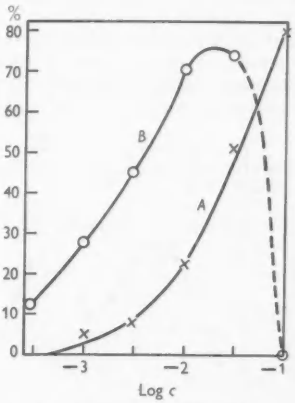

Fig. 1. Effect of varying concentrations of malononitrile on the respiration and aerobic glycolysis of rat-brain slices.

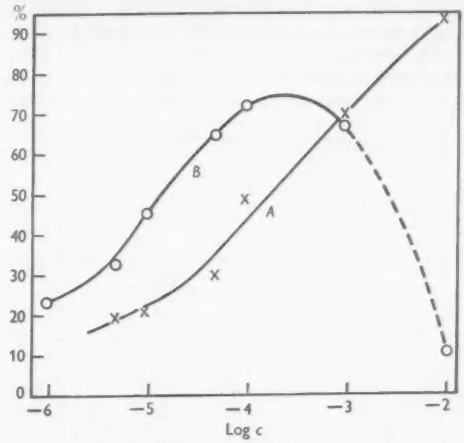

Fig. 2. Effect of varying concentrations of cyanide on the respiration and aerobic glycolysis of rat-brain slices.

Slices suspended in medium III, modified; incubated in O_2 for 1 hr. All points are the means of three to four experiments. Curve *A*: inhibition of respiration. Curve *B*: aerobic glycolysis in percentage of anaerobic glycolysis. *c*, Concentrations of malononitrile and cyanide (M).

acid concentration. It is particularly marked in the last three rats which had received a second injection.

Table 6. *Concentration of lactic acid in brains of rats previously injected with malononitrile*

(All rats received an injection (14 mg./kg.) 2 hr. before sacrifice; rats 13, 14 and 15 received a second injection 1 hr. after the first.)

	Lactic acid (mg./100 g.)	
No.	Found	Excess over mean of control series
1	20·4	−9·0
2	24·9	−4·5
3	29·7	0·3
4	31·0	1·6
5	31·4	2·0
6	47·8	18·4
7	49·4	20·0
8	54·2	24·8
9	72·0	42·6
10	82·5	53·1
11	98·0	68·6
12	122·0	92·6
13	103·0	73·6
14	107·0	77·6
15	112·0	82·6

In fifteen control experiments the mean value was 29·4 mg./100 g., S.D. 7·0, S.E. 1·81. Deviation from mean at $P \leqq 0{\cdot}05$, $\geqq 15{\cdot}0$.

These experiments show that the increase of aerobic glycolysis observed with brain slices also occurs in the living brain after an injection of malononitrile.

The concentration of cozymase in rat brain was not significantly changed after malononitrile injection. The mean value found in twelve control rats was $26{\cdot}74 \pm 1{\cdot}78$ mg./100 g. of fresh brain, that in seventeen injected rats, $24{\cdot}57 \pm 1{\cdot}91$ mg. The probability of the difference being due to chance variation is 0·44. The cozymase concentration found in these experiments is midway between the figures reported by Gore, Ibbott & McIlwain (1950) and by Axelrod & Elvehjem (1939).

The effects of tissues on malononitrile

If malononitrile, together with sodium thiosulphate, is added to a tissue homogenate, a formation of SCN^- is observed which increases linearly with time (Fig. 3). The activities of homogenates of liver, kidney and brain decrease in that order; in the rat they are in the proportion of 34:14:1. Rhodanese shows a very similar distribution; according to Himwich & Saunders (1948), rhodanese activities of rat liver, kidney and brain cortex are as 20–40:15:1. The amounts of SCN^- formed from cyanide by kidney and liver preparations are however 2500–3000 times higher, per unit of weight, than those formed from malononitrile. This comparison is based on our own estimations of rhodanese activity at 30° which, for extracts of rat kidney and liver, gave figures three to four times higher than those reported by Himwich & Saunders. Rhodanese is therefore present in vast excess. Since rhodanese activity is not inhibited by malononitrile in concen-

trations up to 0·02 M (Table 10) it cannot be a limiting factor in the formation of SCN^- from malononitrile.

The system responsible for the formation of SCN^- from malononitrile by rat-liver homogenate in the presence of 0·05 M-sodium thiosulphate is saturated

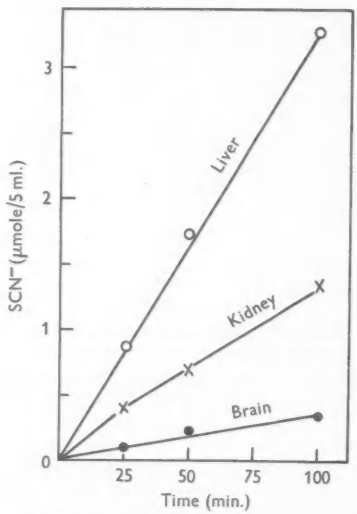

Fig. 3. Formation of thiocyanate from malononitrile (0·005 M) and thiosulphate (0·05 M) by rat tissue homogenates. Final dilution of liver and kidney homogenates 1:33, of brain homogenate 1:9. The medium contained 0·04 M-glycine buffer, pH 8·2. All points were corrected for thiocyanate content of control experiments containing boiled homogenates.

by 0·0033 M-malononitrile. The low concentration level at which saturation occurs is in favour of a specific catalyst.

The pH optimum for the formation of SCN^- from malononitrile by liver extract is near 7·0 (Fig. 4). The activity of the system is higher in glycine than in phosphate buffer. Although phosphate buffer was used in the earlier experiments it was later replaced by a glycine buffer of pH 8·2. The final pH of the reaction mixture which was repeatedly checked with a glass electrode was 7·2–7·3.

Saunders & Himwich (1950) noted that rhodanese is strongly inhibited by cysteine. The same applies to SCN^- formation from malononitrile. Inhibition of a less severe degree was also observed in the presence of reduced glutathione and of adenosinetriphosphate. Sodium fluoride, on the other hand, is without effect (Table 7).

No trace of SCN^- formation was found when any of the following substances, in 0·005 M-concentration, was incubated with rat-liver homogenate (1:33) for 2 hr. at 30°: acetonitrile, propionitrile, butyronitrile, *iso*valeronitrile, succinonitrile, glutaronitrile, dimethylmalononitrile and ethyl cyanoacetate.

The effect of thiosulphate. The action of tissue preparations on malononitrile was further studied in experiments in which CN^- and SCN^- were estimated in separate samples of the same solution

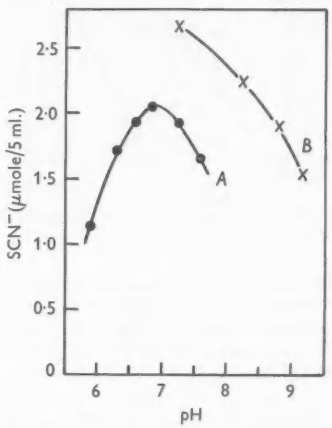

Fig. 4. Effect of pH on thiocyanate formation from malononitrile (0·005 M) by rat-liver extract (1:33). The medium contained $Na_2S_2O_3$ 0·05 M, buffers 0·05 M. Incubated at 30° for 2 hr. Curve *A*: phosphate buffers. Curve *B*: glycine buffers. The values of pH were determined after the incubation with a glass electrode. All points were corrected for the thiocyanate content of control experiments with boiled extract.

Table 7. *Inhibition of thiocyanate formation from malononitrile by rat-liver extract*

(5 ml. solution contained 0·005 M-malononitrile, 0·05 M-$Na_2S_2O_3$, 0·05 M-phosphate buffer pH 7·4, extract 1:16·67. Incubated for 2 hr. at 30°.)

Addition	Rate of SCN^- formation as percentage of control experiment
0·01 M-Cysteine	10·1
0·01 M-Glutathione (GSH)	38·5
0·005 M-Adenosinetriphosphate	68·5
0·03 M-NaF	92

(Table 8). It was usually found that the sum of the molar concentration of the two ions was larger when the incubation was performed in the presence of sodium thiosulphate. The results were corrected for non-enzymic effects by control experiments with boiled tissue preparations. Some non-enzymic formation of CN^- took place; the boiled preparations also seemed to possess a residue of rhodanese activity. The non-enzymic reaction is, however, small compared with the enzymic reaction, especi-

ally in presence of $S_2O_3^{--}$. The results obtained with kidney and brain, though on a smaller scale, were similar to those observed with liver. Liver homogenate was slightly more active than liver extract, but no significant difference was found between homogenate and extract in the case of kidney or brain.

Similar experiments were performed with methyl and ethyl thiocyanate (Table 9). The results are complicated by a considerable non-enzymic formation of cyanide, especially from methyl thiocyanate. $S_2O_3^{--}$ has little effect in presence of a denatured tissue preparation, but it does increase the sum total of $CN^- + SCN^-$ in presence of an enzymically active

Table 8. *Effect of tissue homogenates and extracts on malononitrile* (0·01 M) *in presence and absence of thiosulphate*

(5 ml. solution contained 0·04 M-glycine buffer, pH 8·2. Incubation for 2 hr. at 30°. H, homogenate. E, extract.)

					μmoles								
					(1) Preparation enzymically active			(2) Preparation boiled			Difference (1) – (2)		
No.	Tissue (rat)	Final dilution	Prepara-tion	$Na_2S_2O_3$ (M)	CN^-	SCN^-	Total	CN^-	SCN^-	Total	CN^-	SCN^-	Total
1	Liver	1:33	H	0	0·91	0·46	1·37	0·37	0·26	0·63	0·54	0·20	0·74
				0·05	0·40	2·42	2·82	0·27	0·41	0·68	0·13	2·01	2·14
			E	0	0·96	0·36	1·32	0·37	0·31	0·68	0·59	0·05	0·64
				0·05	0·40	1·75	2·15	0·28	0·41	0·69	0·12	1·34	1·46
2	Liver	1:33	H	0	0·56	0·31	0·87	0·58	0·31	0·89	−0·02	0	−0·02
				0·05	0·15	2·12	2·27	0·47	0·41	0·88	−0·32	1·71	1·39
			E	0	0·54	0·31	0·85	0·50	0·31	0·81	0·04	0	0·04
				0·05	0·19	1·65	1·84	0·56	0·41	0·97	−0·37	1·24	0·87
3	Kidney	1:33	H	0	0·35	0·26	0·61	0·28	0·26	0·54	0·07	0	0·07
				0·05	0·20	1·03	1·23	0·23	0·31	0·54	−0·03	0·72	0·69
			E	0	0·38	0·26	0·64	0·26	0·21	0·47	0·12	0·05	0·17
				0·05	0·28	0·98	1·26	0·21	0·26	0·47	0·07	0·72	0·79
4	Brain	1:16·7	H	0	0·12	0·29	0·41	0·09	0·19	0·28	0·03	0·10	0·13
				0·05	0·03	0·52	0·55	0·08	0·20	0·28	−0·05	0·32	0·27
			E	0	0·14	0·32	0·46	0·08	0·15	0·23	0·06	0·17	0·23
				0·05	0·03	0·57	0·60	0·09	0·19	0·28	−0·06	0·38	0·32

Table 9. *Effect of liver homogenates and extracts on methyl and ethyl thiocyanate*

Arrangement as described in Table 8

(Controls. Exp. 1: control contained boiled tissue preparation and was incubated for 2 hr. at 30°. Exp. 2 and 3: 5 ml. 10% trichloroacetic acid were added to control sample before addition of tissue preparation. The sample was analysed without previous incubation. Me-SCN, methyl thiocyanate; Et-SCN, ethyl thiocyanate; H, homogenate; E, extract.)

				μmoles											
				(1) Preparation enzymically active			(2) Control			(3) Drug incubated without tissue			Difference (1) – (2)		
No.	Prepara-tion	Drug added	$Na_2S_2O_3$ (M)	CN^-	SCN^-	Total	CN^-	SCN^-	Total	CN^-	SCN^-	Total	CN^-	SCN^-	Total
1	H	Me-SCN	0	1·50	0·57	2·07	2·44	0·26	2·70	—	—	—	−0·94	0·31	−0·63
		(0·01 M)	0·05	1·50	1·68	3·18	2·84	0·46	3·30	—	—	—	−1·34	1·22	−0·12
	E		0	1·31	0·41	1·72	1·31	0·21	1·52	—	—	—	0	0·20	0·20
			0·05	1·50	1·50	3·00	1·89	0·37	2·26	—	—	—	−0·39	1·13	0·74
2	H	Me-SCN	0	0·56	0·21	0·77	1·35	0	1·35	1·04	0	1·04	−0·79	0·21	−0·58
		(0·005 M)	0·05	0·60	1·01	1·61	1·35	0·04	1·39	1·53	0	1·53	−0·75	0·97	0·22
	E		0	0·77	0·10	0·87	1·48	0	1·48	—	—	—	−0·71	0·10	−0·61
			0·05	0·70	0·83	1·53	1·30	0·04	1·34	—	—	—	−0·60	0·79	0·19
3	H	Et-SCN	0	0·23	0·52	0·75	0·47	0	0·47	0·21	0	0·21	−0·24	0·52	0·28
		(0·005 M)	0·05	0·35	0·81	1·16	0·58	0	0·58	0·62	0	0·62	−0·23	0·81	0·58
	E		0	0·54	0·46	1·00	0·40	0	0·40	—	—	—	0·14	0·46	0·60
			0·05	0·35	0·77	1·12	0·36	0	0·36	—	—	—	−0·01	0·77	0·76

preparation, and even without any tissue at all. The evidence for an enzymic effect is less ambiguous in the experiment with ethyl thiocyanate; whether SCN^- is formed by direct hydrolysis or by the secondary reaction of the CN group with an S compound cannot be decided, but the increase of SCN^- formation in presence of $S_2O_3^{--}$ is in favour of the second mechanism. A surprising feature, especially of the experiments with methyl thiocyanate, is the relatively high concentration of CN^- which is observed even in the presence of $S_2O_3^{--}$ and with and one without $S_2O_3^{--}$; to a third $S_2O_3^{--}$ was added 10 min. before termination of the experiment. Owing to the large excess of rhodanese activity, it can be expected that 10 min. are sufficient to allow the transformation of any CN^- present into SCN^- after adding $S_2O_3^{--}$. The formation of SCN^- was larger when $S_2O_3^{--}$ was present throughout the experiment than when its addition was delayed. The result was similar with each of the three compounds tested, but it was most marked in the case of malononitrile.

Table 10. *Effect of malononitrile and alkyl thiocyanates on rhodanese activity*

(5 ml. solution contained 0·05 M-KCN, neutralized, 0·05 M-$Na_2S_2O_3$, 0·04 M-glycine buffer pH 8·2, 0·02 M-malononitrile or alkyl thiocyanate. Incubated for 10 min. at 30°.)

Tissue extract (rat)	Final dilution	Activity as percentage of control experiment in presence of: Malononitrile	Methyl thiocyanate	Ethyl thiocyanate
Liver	1:3333	100	60·6	67·2
Kidney	1:1667	100	60·0	63·5
Brain	1:250	100	66·0	74·6

an active rhodanese. This may to some extent be accounted for by an inhibition of rhodanese activity in presence of methyl or ethyl thiocyanate (Table 10).

Table 11. *Effect of delayed addition of thiosulphate on thiocyanate formation from malononitrile, methyl and ethyl thiocyanate by rat-liver homogenate*

(5 ml. solution contained 0·04 M-glycine buffer pH 8·2, malononitrile or alkyl thiocyanate 0·005 M, homogenate 1:33. Incubated for 2 hr. at 30°.)

Addition of 0·05 M-$Na_2S_2O_3$	Formation of SCN^- (μmoles) from: Malononitrile	Methyl thiocyanate	Ethyl thiocyanate
None	0·25	0·26	0·36
At start	1·44	0·75	0·58
After 110 min.	0·55	0·39	0·48

The effect of $S_2O_3^{--}$ was further demonstrated by the following experiment (Table 11): of three samples of liver homogenate one was incubated

For the interpretation of the effect of sodium thiosulphate it is essential to know whether the presence of sodium thiosulphate affects the recovery of the sum total of $CN^- + SCN^-$ after the incubation of CN^- with tissues. In experiments with rat-liver homogenate it was found that added CN^- is quantitatively recovered in the form of SCN^- when $S_2O_3^{--}$ is present, but in its absence the sum of $CN^- + SCN^-$ does not account for the CN^- initially added (Table 12). This result suggests that the effect of $S_2O_3^{--}$ is due, at least in part, to the successful competition of rhodanese with other systems capable of removing CN^-.

Effects of purified rhodanese. Several observations suggest a connexion between rhodanese and the system responsible for the SCN^- formation from malononitrile and alkyl thiocyanates, e.g. the distribution of activity, the dependence on $S_2O_3^{--}$ and the inhibition of cysteine. The action of purified liver rhodanese was therefore investigated. The enzyme was prepared from rabbit liver by the

Table 12. *Recovery of cyanide and thiocyanate after incubation of rat-liver homogenate with cyanide in presence and absence of thiosulphate*

(5 ml. solution contained 150 mg. homogenized rat liver and 0·04 M-glycine buffer, pH 8·2. Final pH adjusted to 7·3. Incubated for 2 hr. at 30°.)

Exp. no.	$Na_2S_2O_3$ (M)	CN^- added	μmoles: Initial recovery (1) CN^-	SCN^-	Total	Final recovery (2) CN^-	SCN^-	Total	Difference (2) − (1) CN^-	SCN^-	Total
1	0	3·85	3·77	0	3·77	2·62	0	2·62	−1·15	0	−1·15
	0·05		3·85	0	3·85	0·03	3·81	3·84	−3·82	+3·81	−0·01
2	0	3·70	3·46	0	3·46	2·85	0·07	2·92	−0·61	+0·07	−0·54
	0·05		3·62	0	3·62	0	3·87	3·87	−3·62	+3·87	+0·25
3	0	3·54	3·39	0	3·39	2·54	0	2·54	−0·85	0	−0·85
	0·05		3·54	0·07	3·62	0	3·87	3·87	−3·54	+3·80	+0·25

method of Cosby & Sumner (1945). The final product, assayed according to these authors, formed 4·5 μmoles SCN^-/mg. protein in 5 min. at 20°. The effect of this preparation on malononitrile, methyl and ethyl thiocyanate was tested in an experiment in which these substances were incubated for 3 hr. at 30° with an amount containing 38 mg. protein. Samples containing boiled enzyme solution served as controls. Ten minutes before the termination of the experiment, active enzyme solution containing 4·2 mg. protein was added to both experimental and control flasks in order to convert any CN^- formed in the control samples to SCN^-. The ratio of activities should remain unchanged after purification; a simple calculation shows that, if the effects of the crude extracts were due to the activity of rhodanese, malononitrile would yield at least 2 μmoles SCN^- (at 20°). Instead, purified rhodanese was without effect on the formation of SCN^- from malononitrile (Table 13). Ethyl and particularly

Table 13. *Effects of purified rhodanese*

(7·5 ml. solution contained 38 mg. protein, 0·05 M-$Na_2S_2O_3$, 0·04 M-glycine buffer pH 8·2. Incubated at 30°. After 3 hr. 0·5 ml. enzyme solution (=4·2 mg. protein) were added to all flasks and incubation was continued for 10 min.)

Additions (0·01 M)	Formation of SCN^- (μmoles)	
	Boiled enzyme	Active enzyme
Malononitrile	0·14	0·14
Methyl thiocyanate	2·45	1·07
Ethyl thiocyanate	1·60	0·56

methyl thiocyanate gave rise to SCN^- formation, but the effect was larger in the sample containing denatured protein and was therefore presumably non-enzymic.

DISCUSSION

The observations here recorded are compatible with the supposition that the pharmacological effects of malononitrile are the consequence of a gradual liberation of cyanide in the tissues. The increase and eventual inhibition of the aerobic glycolysis of tissue slices by increasing concentrations of cyanide is of course well known (cf. Warburg, 1949). As our results with rat-brain slices show, low concentrations of cyanide seem to affect the aerobic glycolysis more strongly than the respiration; the stimulation of aerobic glycolysis reached its peak when the respiration was inhibited by about 50%. Similar results were obtained with malononitrile, though in concentrations about 100 times higher than those of cyanide. The similarity does not necessarily indicate the identity of the inhibitory mechanism, since results of this kind may be observed with a variety of respiratory inhibitors, such as nicotine (Baker, Fazekas & Himwich, 1938), barbiturates (Webb & Elliott, 1951), maleic (Weil-Malherbe, 1938) or cinnamic acid (Weinbach, Lowe, Frisell & Hellerman, 1951). On the other hand, out of a series of nitriles, including dimethylmalononitrile, none except malononitrile showed either cyanide-like effects on tissue metabolism or gave rise to SCN^- formation in presence of tissue preparations. The only substances which in both these respects resembled malononitrile were methyl and ethyl thiocyanate. It is therefore highly probable that the effects on tissue metabolism are correlated with the transformations induced by tissue preparations.

Compared with its antidotal activity *in vivo* the antagonistic effect of sodium thiosulphate against malononitrile is slight and transient in tissue-slice experiments. The discrepancy also applies to cyanide, though to a lesser extent. Rhodanese is known to be localized mainly in the mitochondrial fraction of liver cells (Ludewig & Chanutin, 1950; Archibald, Meyer & Greenough, 1951) and is thus accessible only to intracellular substances; as Himwich & Saunders (1948) have shown, the rhodanese activity of rabbit liver or brain is about sixteen times larger when the tissue is homogenized than when it is sliced. Permeability factors may thus account for the results *in vitro*, though it is obscure why they seem less important *in vivo*.

Whereas a formation of SCN^- could be detected *in vitro* only from malononitrile, the reaction is known to occur with most other nitriles *in vivo*. This difference is probably quantitative rather than qualitative. Next to cyanogen, malononitrile has a more rapid toxic action than other dinitriles in the body (Heymans & Masoin, 1897). Toxic symptoms and SCN^- excretion are slow to appear and to disappear with most nitriles, and it may be assumed that their rate of breakdown is too slow to be demonstrable in a short-term experiment with tissue preparations.

The increase of lactic acid concentration in the brains of injected rats shows that the aerobic glycolysis observed with brain slices also occurs in the intact animal. The results are in line with those obtained in studies of cyanide poisoning (Albaum, Tepperman & Bodansky, 1946; Olsen & Klein, 1947). On the other hand, the cozymase content of rat brain was found to be unaltered after malononitrile injection. The microspectrographic observations of Hydén & Hartelius (1948) are thus not reflected in concentration changes of this particular nucleotide which might be expected to be especially sensitive to changes in respiratory activity.

The effect of $S_2O_3^{--}$ on the formation of SCN^- from malononitrile seems to be adequately explained by the prevention of side reactions in which part of the cyanide liberated from the nitrile may dis-

appear, but the possibility cannot be excluded that $S_2O_3^{--}$ in some way activates the decomposition of malononitrile. Saunders & Himwich (1950) showed that rhodanese, unless protected by $S_2O_3^{--}$, is inhibited by cyanide. Though the concentration of CN^- present in our experiments is too small to make rhodanese activity limiting, the activity of some other enzyme taking part in the reaction may be similarly affected. There is further the possibility that $S_2O_3^{--}$ may be involved in the reaction as a specific electron donor. In favour of such an assumption is the catalytic effect of $S_2O_3^{--}$ on the formation of CN^- from methyl and ethyl thiocyanates in absence of a tissue preparation. The action of tissue preparations on the alkyl thiocyanates may be largely due to an unspecific effect of SH groups, since it persists after denaturation of protein. It is interesting to note that Aldridge (1951) has proposed a mechanism for the dissociation of cyanogen chloride involving complex formation with reduced glutathione. As far as nitriles are concerned it is known that $S_2O_3^{--}$ accelerates the dissociation of the labile lactonitrile (Meurice, 1900). On the other hand, no effect of $S_2O_3^{--}$ on the spontaneous dissociation of malononitrile could be detected at 30° in short-term experiments. Moreover, malononitrile was only attacked by enzymically active, but not by denatured, tissue preparations and this reaction was inhibited by SH compounds. It thus seems that two mechanisms must be distinguished: an unspecific effect which is predominant in the case of alkyl thiocyanates and which may be mediated by SH-groups and a specific, enzymic effect which is predominant in the case of malononitrile.

SUMMARY

1. Out of a series of compounds, including eleven nitriles and ethyl cyanoacetate only malononitrile, methyl thiocyanate and ethyl thiocyanate were found to have any marked effect on tissue slices and to give rise to the formation of cyanide and thiocyanate in presence of tissue homogenates or extracts.

2. In experiments with tissue slices, malononitrile caused an inhibition of respiration, progressive with time, and an increase of aerobic glycolysis. Its action resembled that of cyanide at a molar concentration 100 times smaller. Thiosulphate antagonizes the effects of cyanide to some extent while it alleviates those of malononitrile only in the first, not in the second, hour of the experiment.

3. Subcutaneous injection of malononitrile (14 mg./kg.) increased the lactic acid content of rat brain after 1–2 hr., but did not change the concentration of cozymase.

4. The formation of thiocyanate from malononitrile and thiosulphate is highest in presence of liver tissue, lowest with brain and intermediate with kidney. The liver enzyme system is saturated at a concentration of 0·0033 M-malononitrile and has a pH optimum of 7·0. It is inhibited by cysteine and glutathione and inactivated by boiling.

5. The sum total of cyanide and thiocyanate formed from malononitrile by enzymically active tissue preparations was larger in presence than in absence of thiosulphate. The effect appears to be mainly due to the conversion of cyanide into thiocyanate thus preventing its disappearance in secondary reactions.

6. The formation of cyanide and thiocyanate from methyl and ethyl thiocyanate seems to be largely due to unspecific, non-enzymic factors. Both substances, at 0·02 M-concentration, cause a 40 % inhibition of rhodanese activity.

7. A purified preparation of rhodanese did not induce any formation of thiocyanate from malononitrile and thiosulphate.

A specimen of malononitrile has been put at our disposal by Dr A. Roberts, Medical Department, Messrs Boots Pure Drug Co. Ltd. J. Stern has been working under an assistance grant from the Medical Research Council. We wish to express our gratitude for the help received.

REFERENCES

Albaum, H. G., Tepperman, J. & Bodansky, O. (1946). *J. biol. Chem.* **164**, 45.
Aldridge, W. N. (1951). *Biochem. J.* **48**, 271.
Archibald, R. M., Meyer, R. & Greenough, J. (1951). *Fed. Proc.* **10**, 157.
Axelrod, A. E. & Elvehjem, C. A. (1939). *J. biol. Chem.* **131**, 77.
Baker, Z., Fazekas, J. F. & Himwich, H. E. (1938). *J. biol. Chem.* **125**, 545.
Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Boxer, G. E. & Rickards, J. C. (1951). *Arch. Biochem.* **30**, 372.
Corson, B. B., Scott, R. W. & Vose, C. E. (1932). *Organic Synth.* Coll. vol. **1**, 173.
Corson, B. B., Scott, R. W. & Vose, C. E. (1943). *Organic Synth.* Coll. vol. **2**, 379.
Cosby, E. L. & Sumner, J. B. (1945). *Arch. Biochem.* **7**, 457.
Dickens, F. & Šimer, F. (1931). *Biochem. J.* **25**, 973.
Epstein, J. (1947). *Anal. Chem.* **19**, 272.
Errera, G. (1896). *Gazz. chim. ital.* **26**, no. 1, 197.
Gore, M., Ibbott, F. & McIlwain, H. (1950). *Biochem. J.* **47**, 121.
Heymans, J. F. & Masoin, P. (1897). *Arch. int. Pharmacodyn.* **3**, 77.
Hicks, S. P. (1950). *Arch. Path.* **50**, 545.
Himwich, W. A. & Saunders, J. P. (1948). *Amer. J. Physiol.* **153**, 348.

Hunt, R. (1923). *Handbuch der experimentellen Pharmakologie*, vol. 1, p. 702. Berlin: J. Springer.
Hydén, H. & Hartelius, H. (1948). *Acta psychiat., Kbh.* (Suppl. 48).
Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
Krebs, H. A. (1950). *Biochim. Biophys. Acta, Amst.*, **4**, 249.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Lang, K. (1933). *Biochem. Z.* **259**, 243.
LePage, G. A. (1947). *J. biol. Chem.* **168**, 623.
LePage, G. A. (1948). *Methods in Medical Research*, **1**, 337. Chicago: The Year Book Publishers Inc.
Liu, C. (1951). *Arch. Neurol. Psychiat., Chicago*, **66**, 427.
Ludewig, S. & Chanutin, A. (1950). *Arch. Biochem.* **29**, 441.
Meurice, J. (1900). *Arch. int. Pharmacodyn.* **7**, 1.
Oettingen, W. F. von, Hueper, W. C. & Deichmann-Gruebler, W. (1936). *J. Industr. Hyg.* **18**, 310.
Olsen, N. S. & Klein, J. R. (1947). *J. biol. Chem.* **167**, 739.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Reale, G. & D'Argenio, L. (1951). *Riv. Pat. nerv. ment.* **71**, 171.
Rosenthal, O., Rogers, C. S., Vars, H. M. & Ferguson, C. C. (1950). *J. biol. Chem.* **185**, 669.
Samuels, A. J., Boyarsky, L. L., Gerard, R. W., Libet, B. & Brust, M. (1951). *Amer. J. Physiol.* **164**, 1.
Saunders, J. P. & Himwich, W. A. (1950). *Amer. J. Physiol.* **163**, 404.
Schlenk, F. & Schlenk, T. (1947). *Arch. Biochem.* **14**, 131.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Minneapolis: Burgess Publishing Co.
Warburg, O. (1911). *Verh. dtsch. Kongr. inn. Med.* **28**, 553.
Warburg, O. (1924). *Biochem. Z.* **152**, 51.
Warburg, O. (1949). *Heavy Metal Prosthetic Groups and Enzyme Action.* Oxford: Clarendon Press.
Warburg, O. & Wiesel, R. (1912). *Pflüg. Arch. ges. Physiol.* **144**, 465.
Webb, J. L. & Elliott, K. A. C. (1951). *J. Pharmacol.* **103**, 24.
Weil-Malherbe, H. (1938). *Biochem. J.* **32**, 2257.
Weinbach, E. C., Lowe, H. J., Frisell, W. R. & Hellerman, L. (1951). *J. biol. Chem.* **189**, 779.

The Oxidation of Manganese by Enzyme Systems

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Kenten & Mann (1949, 1950) found that certain plant extracts catalysed the oxidation of bivalent manganese (Mn^{++}) by added hydrogen peroxide and showed that this catalysis was due to peroxidase systems in the plant extracts. It was suggested that *in vivo* hydrogen peroxide formed by flavoprotein enzyme systems could be used for the oxidation of Mn^{++} by peroxidase systems and colorimetric evidence of such oxidation was obtained using the xanthine-oxidase-hypoxanthine system as a source of hydrogen peroxide. In later work, Kenten & Mann (1951) demonstrated that many plant extracts, in the absence of added hydrogen peroxide, oxidized Mn^{++} and that with pea-seedling extracts the oxidation depended on the formation of hydrogen peroxide by an amine oxidase present in the extracts.

In the present work it is shown that manganese oxidation can be brought about by coupling a number of enzyme systems producing hydrogen peroxide with peroxidase systems.

MATERIALS AND METHODS

Peroxidase. This was prepared from horse-radish extracts by precipitation with $(NH_4)_2SO_4$ followed by fractional precipitation with ethanol according to the method of Keilin & Mann (1937). The 'Purpurogallinzahl' (P.Z.) of the preparation used in the present work estimated by the method of Keilin & Mann (1937) was 250.

Catalase. This was prepared from horse liver by a method based on that of Agner (1938). The activity of the preparation was estimated by the method of Sumner & Somers (1943). The decomposition of 0·01 N-H_2O_2 in 0·0067 M-orthophosphate at pH 7 and 0°, by a suitable amount of catalase, was followed by iodine titration on samples taken at 0, 3, 6, 9 and 12 min. From the equation

$$k = (1/t) \log_{10} \{a/(a-x)\},$$

where a is the initial H_2O_2 concentration and $a-x$ is the concentration at time t, k values for the time intervals were calculated and the k value at zero time obtained by extrapolation. Using this value for k the catalase activity $= k/g$. enzyme of the preparations used varied from 5000 to 6000.

Plant amine oxidase. This was prepared from pea seedlings by the method of Kenten & Mann (1951). The dry preparation was suspended in 0·2 M-pyrophosphate buffer at pH 7·5 or 0·2 M-orthophosphate at pH 7 and centrifuged clear before use.

Xanthine oxidase. This was prepared from milk by the method of Dixon & Kodama (1926). The 'whey preparation' was suspended in 0·2 M-pyrophosphate at pH 7·5 and centrifuged before use.

D-*Amino-acid oxidase.* This was prepared from sheep kidney. The method of Negelein & Brömel (1939) was followed as far as the first precipitation with $(NH_4)_2SO_4$. This precipitate was redissolved in 0·0033 M-pyrophosphate

pH 8·3. The precipitate formed on addition of 15 g. $(NH_4)_2SO_4$/100 ml. was removed by centrifuging. The enzyme was precipitated by addition of a further 15 g. $(NH_4)_2SO_4$/100 ml. This was separated by centrifuging and suspended in 100 ml. 0·0033 M-pyrophosphate pH 8·3, kept at 0° overnight and centrifuged clear before use.

α-Hydroxyacid oxidase was prepared from dried leaves of brussels sprouts (*Brassica oleracea* var. *gemmifera* Zenk.). The temperature of the leaf extracts was kept below 5° throughout the preparation except during centrifuging. The final preparations were stored frozen. The small outside leaves of the axillary buds ('sprouts') were oven dried at 37°. The dried leaves were powdered in a mortar and passed through a 0·5 mm. mesh sieve. The dry powder could be stored for several weeks at room temperature with little loss of activity. For the enzyme preparation 15 g. samples were suspended in 300 ml. 0·1 M-phosphate at pH 8 and 0° and treated for a few minutes in a Townson & Mercer macerator, cooling when necessary to prevent the temperature rising above 5°. The suspension was then squeezed through madapollam and an α-hydroxyacid oxidase preparation was made from the extract so obtained by the procedure of Clagett, Tolbert & Burris (1949) up to the $(NH_4)_2SO_4$ fractionation. The fraction precipitating between 10 g. and 40 g. $(NH_4)_2SO_4$/100 ml. was dissolved in water and purified by treatment with $Ca_3(PO_4)_2$ gel at pH 8. The gel was prepared by the method of Singer & Kearney (1950) and had a dry weight of 20 mg./ml. The amounts of gel required varied with the different preparations. In general, 0·5–1·0 ml. gel/20 ml. enzyme solution was added and the suspension centrifuged at once. To the supernatant a further 2–5 ml. gel were added and the suspension left at 0° for 30 min. and then centrifuged. The gel was then suspended in 10 ml. 0·2 M-pyrophosphate buffer at pH 7·5 left 30 min. at 0° and then centrifuged. The clear supernatant fluid contained about 30 % of the activity of the $(NH_4)_2SO_4$ fraction towards DL-sodium lactate. Clagett *et al.* (1949) found that $Ca_3(PO_4)_2$ gel adsorbed the enzyme at pH 8, but no elution was achieved with orthophosphate. The elution obtained with pyrophosphate at pH 7·5 may be due to its solvent action on the gel surface.

Manometric measurements. These were made in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. and KOH was present in the centre cup. The gas space was air unless otherwise stated.

EXPERIMENTAL AND RESULTS

Experiments in pyrophosphate with D-amino-acid oxidase, plant amine oxidase and xanthine oxidase

The reactions catalysed by amine oxidase (Kenten & Mann, 1951), D-amino-acid oxidase (Keilin & Hartree, 1936) and xanthine oxidase (Thurlow, 1925) proceed according to the general equation

$$RH_2 + O_2 \rightarrow R + H_2O_2. \quad (1)$$

If the H_2O_2 formed is decomposed by catalase the apparent O_2 uptake is halved and the net reaction is

$$RH_2 + \tfrac{1}{2}O_2 \rightarrow R + H_2O. \quad (2)$$

It is well known that H_2O_2 so produced may be used for secondary or coupled oxidations by peroxidase (Thurlow, 1925; Harrison & Thurlow, 1926). Where catalase is present and the primary reaction is proceeding according to Eqn. (2) such coupled oxidation causes increases in the O_2 uptake which may reach 100 % if all the H_2O_2 produced is used in the coupled oxidations. Results obtained with D-amino-acid oxidase are shown in Fig. 1. In this experiment the reaction mixtures in 0·17 M-pyrophosphate, pH 7·5, consisted of D-amino-acid oxidase, 0·2 ml. 0·1 M-DL-alanine, 100 μg. catalase and, where present, 100 μg. peroxidase, 0·3 ml. 0·001 M-*p*-cresol and 0·25 ml. 0·1 M-$MnSO_4$. In the absence of Mn^{++} the O_2 uptake corresponded to slightly more than 1 atom O_2 (Eqn. 2). In presence of Mn^{++} the rate of O_2 uptake was doubled and the total uptake corresponded to 1 mol. O_2/1 mol. D-alanine (Eqn. 1) provided both *p*-cresol and peroxidase were also present. At the end of the experiment this reaction mixture was red, indicating accumulation of manganipyrophosphate. The fact that the O_2 uptake was doubled suggests that the H_2O_2 formed by the D-amino-acid oxidase system was used quantitatively by the peroxidase system to oxidize Mn^{++}. This was further investigated in similar experiments with amine oxidase, D-amino-acid oxidase and xanthine oxidase by not only measuring the O_2 uptakes but also the manganipyrophosphate which accumulated. The manganipyrophosphate was estimated manometrically with hydrazine (Kenten & Mann, 1949).

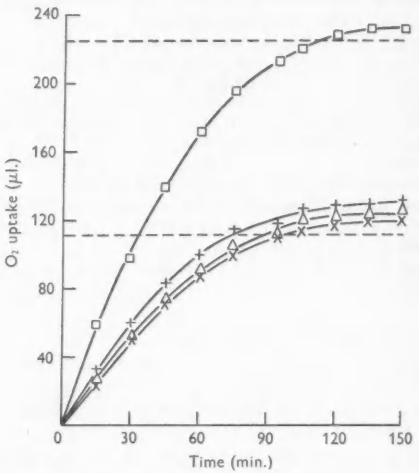

Fig. 1. The oxidation of Mn^{++} by D-amino-acid oxidase systems coupled with peroxidase systems. □—□, D-amino-acid oxidase + DL-alanine + peroxidase + *p*-cresol + Mn^{++} + catalase; +—+, Mn^{++} omitted; △—△, *p*-cresol omitted; ×—×, peroxidase omitted or *p*-cresol and Mn^{++} omitted. Pyrophosphate buffer pH 7·5. The dotted lines represent the theoretical uptakes.

The results of these experiments are shown in Table 1. The amounts of putrescine, D-alanine and hypoxanthine used should give a theoretical uptake of 112 μl. O_2 when oxidized by their respective oxidases in presence of catalase. The values obtained (*A*) were slightly higher than this. The addition of Mn^{++} alone (*B*) produced small increases in the O_2 uptake particularly with the hypoxanthine-xanthine-oxidase system. The addition of *p*-cresol alone (*C*) had little effect on the total O_2 uptake. Where both Mn^{++} and *p*-cresol were added, however, the O_2 uptakes were markedly increased. The values obtained with the D-amino-acid oxidase and xanthine oxidase systems agreed with the theoretical 224 μl. O_2 required by Eqn. (1), but were significantly above this with the amine oxidase system. The doubling of the O_2 uptake is due to the fact that the H_2O_2 produced in the basal reaction is not decomposed by the catalase present but is used by the peroxidase system to oxidize the added Mn^{++}.

If all the H_2O_2 is used to oxidize Mn^{++} and the oxidized manganese accumulated quantitatively then an output of 112 μl. N_2 would be expected on subsequent addition of hydrazine. The N_2 outputs with hydrazine using the amine oxidase and amino-acid oxidase systems agree closely with this theoretical value. The low value with the xanthine oxidase system is due, at least in part, to the fact that manganipyrophosphate oxidizes the uric acid which is formed by the action of the xanthine oxidase on the hypoxanthine, and thereby is itself reduced. It is clear from these results that H_2O_2 formed by enzyme systems can be used quantitatively by peroxidase for the oxidation of Mn^{++}.

Experiments in pyrophosphate with α-hydroxyacid oxidase

Kenten & Mann (1951) found that many plant extracts oxidized added Mn^{++} and suggested that this oxidation was a test for the presence in the extracts of enzyme systems producing H_2O_2. With some extracts addition of lactic acid increased the accumulation of oxidized manganese suggesting that during the oxidation of lactic acid by these extracts H_2O_2 was produced. It is known that certain α-hydroxyacids are oxidized by extracts of the leaves of many plants. Kolesnikov (1948*a*) found that extracts of barley leaves oxidized glycollic acid and Clagett *et al.* (1949) showed the presence in plant leaves of an α-hydroxyacid oxidase catalysing the oxidation of both lactic and glycollic acids. More recent work, Tolbert & Burris (1950), indicates that different enzymes may be responsible for the oxidation of lactic and glycollic acids. While considerations of the total O_2 uptake suggest that H_2O_2 is formed during the oxidations. Tolbert & Burris (1950) were unable to demonstrate its presence and, like Kolesnikov (1948*b*) suggested that an organic peroxide might be formed. Since the preliminary experiments of Kenten & Mann (1951) suggested that the α-hydroxyacid oxidase system might be suitable for the demonstration of coupled oxidation of Mn^{++}, preparations of this enzyme were tested under the conditions of Table 1. The amounts of substrate were chosen so that in absence of coupled oxidation the theoretical uptake was 112 μl. O_2 with purified enzyme preparations; the uptake with L-lactic acid is 1 atom O/mol. L-lactic acid, and with glycollic acid 1 mol. O_2/mol. glycollic acid (Clagett *et al.* 1949). The uptakes with

Table 1. *Oxidation of* Mn^{++} *by oxidase systems coupled with peroxidase systems*

(The basal reaction mixtures (*A*) consisted of oxidase, substrate, 100 μg. catalase, 100 μg. peroxidase in pyrophosphate at pH 7·5 or orthophosphate at pH 7. Other reaction mixtures contained, in addition, 0·25 ml. 0·1 M-$MnSO_4$ (*B*), 0·3 ml. 0·001 M-*p*-cresol (*C*) or both $MnSO_4$ and *p*-cresol (*D*). The oxidase substrate was added from the side arm after equilibration. When the O_2 uptake stopped the manometers were gassed with N_2 and 0·2 ml. saturated $N_2H_4.H_2SO_4$ was added from the side arm and the subsequent N_2 output measured.)

Enzyme	Substrate	Total O_2 uptake (μl.)				N_2 output with hydrazine (μl.)*			
		A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Experiments in pyrophosphate:									
Amine oxidase	Putrescine (10 μmoles)	124	136	127	278	0	8	0	107
D-Amino-acid oxidase	DL-Alanine (20 μmoles)	119	127	131	234	3	13	2	113
Xanthine oxidase	Hypoxanthine (5 μmoles)	127	156	125	237	4	7	4	63
α-Hydroxyacid oxidase	DL-Lactic acid (20 μmoles)	107	111	103	181	2	9	3	83
	Glycollic acid (5 μmoles)	102	104	103	157	4	14	3	70
Experiment in orthophosphate:									
Amine oxidase	Putrescine (5 μmoles)	59	59	66	115	1	6	1	81

* Corrected for 105% recovery (see Kenten & Mann, 1949.)

both substrates (*A*) were slightly less than the theoretical value, particularly with glycollic acid. With glycollic acid the oxidation proceeds in two stages (Clagett *et al.* 1949). The oxidation of glycollic acid to glyoxylic acid which constitutes the first stage is rapid in comparison with the further oxidation of glyoxylic acid to formic acid. Addition of Mn^{++} and *p*-cresol (*D*) produced marked increases in the O_2 uptakes which with lactic acid approached the theoretical doubling while with glycollic acid the increase was approximately 50 %. The N_2 outputs with hydrazine show that these increases in O_2 uptake were due to the oxidation of Mn^{++} and accumulation of manganipyrophosphate. It is clear therefore that a peroxide which can be used by peroxidase to oxidize Mn^{++}, and which is decomposed by catalase, is formed during the oxidation of both lactic and glycollic acids by α-hydroxyacid oxidase.

Experiments in orthophosphate with amine oxidase

Accumulation of manganese oxidation product. In previous work (Kenten & Mann, 1949, 1950) where the oxidation of Mn^{++} was brought about by peroxidase systems with added H_2O_2, it was found that in orthophosphate media the manganese oxidation product reacted with the H_2O_2 as rapidly as it was formed and therefore did not accumulate. In media where stable manganic complexes could be formed, such as pyrophosphate, the oxidation product accumulated though not quantitatively. To explain these results it was shown that manganipyrophosphate reacts less rapidly than MnO_2 with H_2O_2. It was found that the amount of accumulated manganipyrophosphate, calculated as a percentage of that theoretically possible, increased with decreasing H_2O_2 concentration, and it was suggested that at sufficiently low H_2O_2 concentrations all the H_2O_2 would be used in the accumulation of manganipyrophosphate. It has been shown in the experiments of Table 1 that where enzyme systems are used to provide this low concentration of H_2O_2 manganipyrophosphate may accumulate quantitatively. With these systems accumulation of manganese oxidation products can also be demonstrated in orthophosphate media (Table 1). The doubling of the O_2 uptake produced by the addition of Mn^{++} suggested that none of the manganese oxidation product reacts with the H_2O_2 under these conditions. It was found, however, that the hydrazine method was unsuitable to prove that the accumulation was quantitative. The oxidation product, which was present as a red-brown precipitate, reacted much more slowly than manganipyrophosphate or hydrated MnO_2 with the hydrazine, but finally gave a total output greater than that required by theory. The reason for this is not yet clear since MnO_2 reacts quantitatively with hydrazine in orthophosphate media.

Experiments with catalytic amounts of manganese. In the preceding experiments designed to demonstrate quantitative accumulation of manganese oxidation product, Mn^{++} was added in amounts more than equivalent to the H_2O_2 formed by the primary enzyme system, and was therefore present in relatively high concentration. Attempts were made to show that Mn^{++} was oxidized, when present in low concentrations, by adding to the reaction

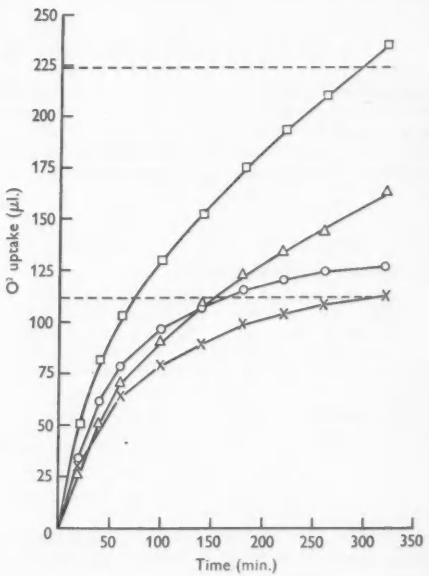

Fig. 2. The oxidation of Mn^{++} by an amine oxidase system coupled with a peroxidase system demonstrated by the oxidation of added oxalic acid. □—□, Amine oxidase + cadaverine + peroxidase + catalase + *p*-cresol + Mn^{++} + oxalic acid; △—△, *p*-cresol omitted; ×—×, Mn^{++} omitted; ○—○, *p*-cresol and Mn^{++} omitted. Orthophosphate buffer pH 5. The dotted lines represent the theoretical O_2 uptakes.

mixtures compounds likely to reduce the oxidized manganese as fast as it was formed. The oxidation of such small amounts of Mn^{++} with accumulation of the oxidation product would produce little increase in the O_2 uptake. If, however, the Mn^{++} entered a cycle of oxidation and reduction, then such amounts would be capable of producing large increases in the O_2 uptake. This was tested with 110 μg. Mn^{++} using the amine oxidase system as source of H_2O_2 and oxalic acid as reductant of the oxidized manganese. The results of one such experiment are shown in Fig. 2. Reaction mixtures in 0·067 M-orthophos-

phate at pH 5 consisted of amine oxidase (4 mg.), 0·2 ml. 0·05 M-cadaverine, 100 μg. catalase, 100 μg. peroxidase, 0·3 ml. 0·001 M-*p*-cresol, 0·2 ml. 0·01 M-$MnSO_4$ and 0·5 ml. 0·1 M-sodium oxalate. The cadaverine was added from the side arm after equilibration. As expected, in the presence of both Mn^{++} and *p*-cresol, an increase in O_2 uptake was observed which was much greater than that required for the complete oxidation of the added Mn^{++} to MnO_2 (22·4 μl. O_2). It is clear therefore that the Mn^{++} undergoes a cycle of oxidation and reduction, and that the observed increase in O_2 uptake is mainly due to the oxidation of the oxalic acid brought about by the oxidized manganese; but the results obtained suggest the possibility that reactions other than the coupled oxidation of the Mn^{++} by the enzyme system and reduction of the oxidation product by the oxalic acid take place. Thus if all the H_2O_2 produced in the oxidation of the cadaverine was used for oxalic acid oxidation via the Mn^{++}, a total O_2 uptake of 224 μl. (1 mol. O_2/mol. cadaverine) would be expected (Eqn. 1). At the end of the experiment the O_2 uptake in the reaction mixture containing Mn^{++} and *p*-cresol was not complete but already exceeded 224 μl. Also, in the absence of *p*-cresol, Mn^{++} alone caused a marked increase in the O_2 uptake in the later stages of the experiment. These observations have led to a separate investigation of reactions involving peroxidase systems, Mn^{++} and oxalic acid.

DISCUSSION

The results of the present work show that, in presence of a system which maintains a suitably low concentration of H_2O_2, peroxidase systems can bring about oxidation of Mn^{++} with quantitative accumulation of the oxidation product. In contrast to previous results with added H_2O_2 (Kenten & Mann, 1950) accumulation of the Mn oxidation product can be demonstrated in orthophosphate as well as in pyrophosphate media. It is clear from these results that, provided the H_2O_2 concentration is sufficiently low, the rate of reduction of the oxidized Mn by the H_2O_2 is negligible. Under these conditions all the oxidized Mn would therefore be available in the plant for the oxidation of metabolites other than H_2O_2, as postulated by Kenten & Mann (1949), and the Mn^{++} would undergo a cycle of oxidation and reduction as demonstrated in the present work with oxalic acid as a reducing agent.

This oxidation of Mn^{++} has not yet been demonstrated in the plant *in vivo* under normal conditions of manganese supply. In preliminary experiments with plants grown in water culture with high manganese concentrations, evidence has been obtained of the accumulation of oxidized Mn in the tissues. Such accumulation can generally be shown in the pea plant (*Pisum sativum* L.) with concentrations of Mn^{++} of the order of 50 p.p.m. in the culture solution. Despite the sensitivity of the test used it has not yet been possible to demonstrate such accumulation regularly with more normal conditions of Mn supply. The results suggest, however, that Mn oxidation does occur within the plant *in vivo* and, in view of previous results with plant extracts (Kenten & Mann, 1951), it is reasonable to postulate that this oxidation is brought about by the peroxidase system. It has already been shown that the oxidation takes place *in vitro* with physiological concentrations of Mn^{++}. At these concentrations of Mn^{++} *in vivo* it is possible that the Mn oxidation product is reduced by plant metabolites as fast as it is formed, and that only under conditions of Mn toxicity is the rate of oxidation of Mn greater than the rate of reduction of the oxidation product by plant metabolites. This suggests the possibility that the symptoms of Mn toxicity may be due, at least in part, to the deposition of higher oxides of manganese in the tissues. Studies with enzyme preparations suggest that Mn^{++} ions may function as activators of many enzyme systems *in vivo*. In general, such effects are not specific for Mn^{++} ions, but to a greater or lesser extent are shared by other bivalent cations. It has not yet been ascertained how far the activity of such enzymes is affected in Mn deficiency, but it may prove that *in vivo* Mn^{++} ions function as specific activators of one or more enzyme systems. It is therefore not suggested that the effects of Mn on plant metabolism depend entirely on the oxidation-reduction cycle postulated. However, an effect of plant metabolism due to such a cycle would be one of some specificity for Mn since, of the bivalent cations tested, peroxidase systems only catalyse the oxidation of Fe^{++} and Mn^{++}.

Lastly it should be pointed out that, as suggested by Kenten & Mann (1951), the oxidation of Mn^{++} by peroxidase systems serves as a useful test for enzyme systems producing H_2O_2. In the present work it is shown by this means that α-hydroxyacid oxidase produces H_2O_2 when catalysing the oxidation of lactic and glycollic acids. This finding has now been confirmed by more conventional methods (Kenten & Mann, 1952).

SUMMARY

1. The oxidation of bivalent manganese (Mn^{++}) can be brought about by enzyme systems producing hydrogen peroxide such as D-amino-acid oxidase, xanthine oxidase and plant amine oxidase systems coupled with peroxidase systems.

2. With high Mn^{++} concentrations the oxidation was demonstrated by the increased oxygen uptake

and by estimation of the manganese oxidation product with hydrazine. The oxidation product accumulates in both pyrophosphate and orthophosphate media.

3. With low Mn^{++} concentrations the oxidation was demonstrated by the increased oxygen uptake of the system in presence of oxalic acid. Under these conditions the manganese is involved in a cycle of oxidation and reduction.

4. The oxidation of Mn^{++} by peroxidase systems can be used as a test for hydrogen peroxide. By this test it has been shown that plant α-hydroxyacid oxidase produces hydrogen peroxide while catalysing the oxidation of L-lactic and glycollic acids.

REFERENCES

Agner, K. (1938). *Biochem. J.* **32**, 1702.
Clagett, C. O., Tolbert, N. E. & Burris, R. H. (1949). *J. biol. Chem.* **178**, 977.
Dixon, M. & Kodama, K. (1926). *Biochem. J.* **20**, 1104.
Harrison, D. C. & Thurlow, S. (1926). *Biochem. J.* **20**, 217.
Keilin, D. & Hartree, E. F. (1936). *Proc. roy. Soc.* B, **119**, 141.
Keilin, D. & Mann, T. (1937). *Proc. roy. Soc.* B, **122**, 119.
Kenten, R. H. & Mann, P. J. G. (1949). *Biochem. J.* **45**, 255.
Kenten, R. H. & Mann, P. J. G. (1950). *Biochem. J.* **46**, 67.
Kenten, R. H. & Mann, P. J. G. (1951). *Biochem. J.* **50**, 360.
Kenten, R. H. & Mann, P. J. G. (1952). *Biochem. J.* **52**, 130.
Kolesnikov, P. A. (1948*a*). *Biokhimiya*, **13**, 370. Cited in *Chem. Abstr.* (1948), **42**, 8889*b*.
Kolesnikov, P. A. (1948*b*). *Dokl. Akad. Nauk. S.S.S.R.* **60**, 1205. Cited in *Chem. Abstr.* (1948), **42**, 7373*f*.
Negelein, E. & Brömel, H. (1939). *Biochem. Z.* **300**, 225.
Singer, T. P. & Kearney, E. B. (1950). *Arch. Biochem.* **29**, 190.
Sumner, J. B. & Somers, G. F. (1943). *Chemistry and Methods of Enzymes*, p. 171. New York: Academic Press, Inc.
Thurlow, S. (1925). *Biochem. J.* **19**, 175.
Tolbert, N. E. & Burris, R. H. (1950). *J. biol. Chem.* **186** 791.

Hydrogen Peroxide Formation in Oxidations Catalysed by Plant α-Hydroxyacid Oxidase

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Kolesnikov (1948*a*) found that extracts of barley leaves oxidized glycollic acid, and Clagett, Tolbert & Burris (1949) showed the presence in leaves of many plants of an α-hydroxyacid oxidase catalysing the oxidation of lactic, glycollic and glyoxylic acids. More recent work (Tolbert & Burris, 1950) indicates that different enzymes may be responsible for the oxidation of these acids. While considerations of the total oxygen uptake suggested that hydrogen peroxide is formed during the oxidations, Tolbert & Burris (1950) were unable to demonstrate its presence, and like Kolesnikov (1948*b*) suggested that an organic peroxide might be formed. Kenten & Mann (1952) demonstrated the oxidation of manganese by flavoprotein enzyme systems coupled with peroxidase systems. It was found that α-hydroxyacid oxidase could replace the known flavoprotein enzymes in producing systems capable of oxidizing manganese, and it was concluded that this was due to the formation of hydrogen peroxide by α-hydroxyacid oxidase systems. In the present work this hydrogen peroxide formation is demonstrated by more conventional methods and its influence on the course of the oxidations is investigated.

MATERIALS AND METHODS

α-Hydroxyacid oxidase. This was prepared from leaves of tobacco (*Nicotiana tabacum* L.). The leaves were minced in a cooled domestic meat mincer and squeezed through madapollam. The temperature of the sap was kept below 5° during the subsequent procedure except during centrifuging. Sufficient 2M-K_2HPO_4 was added to the sap to give a final concentration of 0·1M. The procedure of Clagett *et al.* (1949) was then followed up to and including the first $(NH_4)_2SO_4$ fractionation. The active precipitate from this fractionation was dissolved in water (25 ml./100 ml. original sap) and either dialysed against 0·01M-phosphate buffer at pH 7 overnight in the refrigerator or treated with $Ca_3(PO_4)_2$ gel to reduce the catalase content. This treatment was based on the observation of Agner (1938) that catalase is adsorbed by the gel at pH 5·5. The gel was made by the method of Singer & Kearney (1950) and had a dry weight of 20 mg./ml. The enzyme solution was treated successively with small amounts of gel at pH 5·5. After each addition the suspension was left 30 min. at 0° and then centrifuged. The supernatant was then tested for α-hydroxyacid oxidase and catalase. The amounts of gel required varied with the different enzyme preparations. In one case 20 ml. enzyme solution were treated with 4 ml. of gel followed by two treatments with 1 ml. The catalase activity was then 2%, and the α-hydroxyacid oxidase activity 50%, of that before gel treatment. In

some cases the α-hydroxyacid oxidase was subsequently adsorbed on the gel at pH 8 and eluted with 0·2 M-pyrophosphate buffer at pH 7·5 as described by Kenten & Mann (1952). The preparations were stored frozen.

Catalase. This was prepared from horse liver by a method based on that of Agner (1938). The activity of the preparations was estimated by the method of Sumner & Somers (1943). The decomposition of 0·01 N-H_2O_2 in 0·0067 M-orthophosphate at pH 7 at 0° by a suitable amount of catalase was followed by iodine titration on samples taken at 0, 3, 6, 9 and 12 min. From the equation $k = (1/t) \log_{10} \{a/(a-x)\}$, where a is the initial H_2O_2 concentration and $a - x$ is the concentration at time t, k values for the time intervals were calculated and the k value at zero time obtained by extrapolation. Using this value for k the catalase-activity $= k/g$. enzyme of the preparations used varied from 5000 to 6000. For each experiment a sample of the preparation was suspended in 0·2 M-phosphate buffer (10 mg./ml. buffer) at the required pH and the insoluble material removed by centrifuging. A considerable purification was effected by this procedure.

Carbonyl compounds. These were estimated by the bisulphite method of Clift & Cook (1932).

Formic acid. This was estimated by the method of Pirie (1946).

Manometric measurements. These were made in the Warburg apparatus at 25°. The volume of the reaction mixtures was 3 ml. O_2 uptakes were measured directly with KOH in the centre cups. CO_2 outputs were measured by Warburg's direct method (Dixon, 1943).

EXPERIMENTAL AND RESULTS

Glycollic acid

Clagett *et al.* (1949) found that their preparations of α-hydroxyacid oxidase decomposed H_2O_2 rapidly and that added catalase did not affect the O_2 uptake with glycollic acid as substrate. According to Tolbert, Clagett & Burris (1949) the oxidation of glycollic acid proceeds in two stages through glyoxylic acid to formic acid. In the present experiments using small amounts of enzyme to keep the catalase activity low and working at pH 8·5, which is close to the optimum for the oxidation of glycollic to glyoxylic acid, the enzyme-catalysed reaction studied is mainly the oxidation of glycollic acid to glyoxylic acid. Under these conditions it is possible to demonstrate an effect of added catalase using the dialysed ammonium sulphate preparation of the oxidase (Fig. 1). The composition of the reaction mixtures is given in Table 1. In presence of added catalase the O_2 uptake is rapid until the total uptake approaches 1 atom O/mol. glycollic acid. This rapid stage is mainly due to the oxidation of glycollic acid to glyoxylic acid and is followed by a slow uptake due to the oxidation of the glyoxylic acid. In absence of added catalase the rate of O_2 uptake is

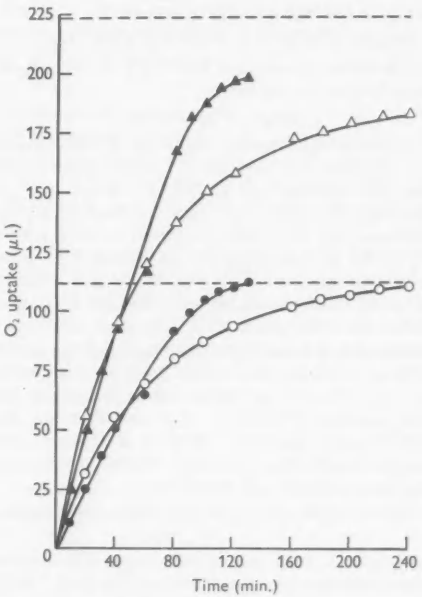

Fig. 1. The effect of catalase on the oxidation of lactate and glycollate by α-hydroxyacid oxidase. △—△, lactate, no added catalase; ○—○, lactate + catalase; ▲—▲, glycollate, no added catalase; ●—●, glycollate + catalase. The dotted lines show the theoretical uptakes.

Table 1. O_2 *uptake and formation of* CO_2 *and other reaction products during the oxidation of lactate glycollate and glyoxylate by α-hydroxyacid oxidase*

(With DL-lactate as substrate the gel-purified preparation of α-hydroxyacid oxidase was used in 0·033 M-pyrophosphate buffer at pH 7·5. With glycollate and glyoxylate the dialysed ammonium sulphate precipitate was used in 0·033 M-orthophosphate buffers at pH 8·5 and 7·7 respectively. Catalase (where present) 0·1 ml.)

Substrate	Catalase	O_2 uptake (μmoles)	CO_2 output (μmoles)	Keto acid (μmoles)	Formic acid (μmoles)
DL-Lactate (10 μmoles)	–	8·2	6·8	2·0	.
	+	5·1	0·6	8·5	.
Glycollate (10 μmoles)	–	8·9	7·5	1·3	.
	+	5·0	0·7	8·7	.
Glyoxylate (20 μmoles)	–	9·3	7·8	.	6·8
	+	8·0	0·7	.	1·4

double that in its presence and proceeds rapidly until the total O_2 uptake approaches 1 mol. O_2/mol. glycollic acid. In the experiment with glycollic acid shown in Fig. 1 the reactions were stopped when the O_2 uptake in presence of catalase was 1 atom O/mol. glycollic acid. The results suggest that the reaction proceeds according to the equation

$$CH_2OH.COOH + O_2 \rightarrow CHO.COOH + H_2O_2. \quad (1)$$

In presence of catalase the H_2O_2 is decomposed according to the equation

$$2H_2O_2 \rightarrow 2H_2O + O_2. \quad (2)$$

Analysis of the reaction mixtures (Table 1) showed that in presence of catalase 87 % of the glycollic acid was now present as carbonyl compounds, presumably glyoxylic acid, while in absence of added catalase only 13 % was accounted for in this form. H_2O_2 did not accumulate (in absence of added catalase) since no evolution of O_2 was observed when catalase was added from the side arms at the end of the experiment. The H_2O_2 must therefore be removed in a secondary reaction. The low value for carbonyl compounds coupled with the high output of CO_2 in absence of added catalase suggests that this secondary reaction may be between the H_2O_2 and the glyoxylic acid. Hatcher & Holden (1925) showed that glyoxylic acid reacts rapidly with H_2O_2 according to the equation

$$CHO.COOH + H_2O_2 \rightarrow CO_2 + H_2O + H.COOH. \quad (3)$$

Solutions of the sodium salt reacted in this way more rapidly than those of the free acid. In the present work it has been found that the reaction proceeds rapidly in phosphate buffers at pH's 7·7 and 8·5. The results therefore suggest that the oxidation of glycollate catalysed by α-hydroxyacid oxidase proceeds according to Eqns. (1) and (2) in presence of added catalase and according to Eqns. (1) and (3) in its absence.

Glyoxylic acid

The oxidation of glyoxylic acid is catalysed by the α-hydroxyacid oxidase preparations at a much slower rate than that of glycollic acid. Despite the fact that larger amounts of the enzyme are therefore required for this oxidation, it is possible to show an effect of added catalase on the rate of O_2 uptake using the dialysed ammonium sulphate preparations (Fig. 2). The composition of the reaction mixtures is given in Table 1. The effect of catalase is not as marked as with glycollic acid as substrate. In the early stages of the reaction the uptake in absence of added catalase is about 50 % higher than in its presence, but the percentage difference decreases with time, and in both cases the O_2 uptake eventually approaches 1 atom O/mol. glyoxylic acid. The effect of added catalase was not more marked with enzyme preparations purified by treatment with calcium phosphate gel to reduce the catalase activity. Hydrogen peroxide does not accumulate since no evolution of O_2 was observed when catalase was added from the side arm at the end of the experiments. In absence of added catalase the CO_2 output was 84 % of that required if the H_2O_2 produced reacted with glyoxylic acid according to Eqn. (3), and formic acid estimations showed the

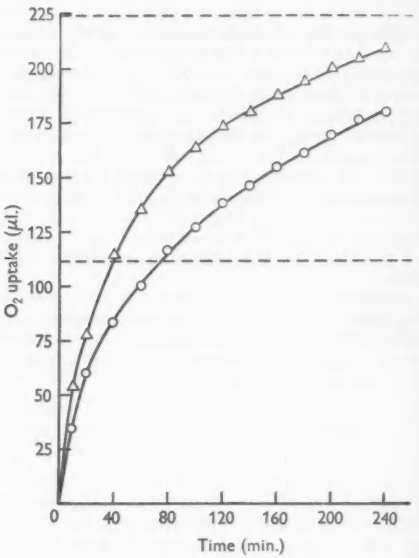

Fig. 2. The effect of catalase on the oxidation of glyoxylate by α-hydroxyacid oxidase. △—△, no added catalase; ○—○, catalase added. The dotted lines show the theoretical uptakes.

presence of 87 % of the formic acid expected based on the CO_2 output (Table 1). In presence of added catalase the CO_2 output was only 9 % and the formic acid found only 20 % of that in absence of added catalase. The results show that the oxidation of glyoxylic acid catalysed by α-hydroxyacid oxidase is accompanied by the formation of H_2O_2. In absence of catalase this H_2O_2 reacts with the substrate to give formic acid and CO_2. This secondary reaction accounts for the increased rate of O_2 uptake in the early stages of the oxidation. It does not produce a doubling of the rate of uptake since it also lowers the concentration of glyoxylic acid available for the enzyme-catalysed reaction. It is probable that the enzyme reaction is between glyoxylic acid monohydrate and O_2 according to the equation

$$\begin{array}{l} COOH + O_2 \\ | \\ CH(OH)_2 \end{array} \rightarrow \begin{array}{l} COOH + H_2O_2 \\ | \\ COOH \end{array} . \quad (4)$$

In the presence of catalase the net reaction should be the sum of Eqns. (4) and (2) and in absence of catalase the sum of Eqns. (4) and (3). In both cases, therefore, the total O_2 uptake should be 1 atom O/mol. glyoxylic acid. In the present experiments the O_2 uptake normally reaches 90–100% of this value in the absence of added catalase but only 70–80% in its presence (Fig. 2). This would be expected if oxalic acid is in fact the product of the enzyme-catalysed reactions, since Tolbert *et al.* (1949) have shown that oxalic acid competitively inhibits the oxidation of glyoxylic acid by the enzyme.

Evidence that oxalic acid is the product of the enzyme-catalysed reaction was obtained by its isolation from a large-scale reaction mixture in presence of added catalase.

A reaction mixture containing 15 ml. 0·1 M-glyoxylate, 5 ml. catalase, 50 ml. of a dialysed preparation of α-hydroxyacid oxidase and 75 ml. 0·2 M-orthophosphate buffer at pH 7·7 in a total volume of 225 ml. was shaken in air at room temperature for 5 hr. The disappearance of glyoxylate was followed by bisulphite titrations on 1 ml. samples at hourly intervals. After 5 hr. 42% of the glyoxylate had disappeared according to the bisulphite titrations. The reaction mixture was then heated at 100° for 5 min., acidified with 2 N-acetic acid to pH 4 and centrifuged. The supernatant was adjusted to pH 5·5 with 8 N-NH_4OH and 20 ml. 20% (w/v) $CaCl_2.6H_2O$ were added. The mixture was allowed to stand overnight and was then centrifuged. The precipitate was dried at 100°, powdered, and the oxalic acid extracted by the method of Pucher, Vickery & Wakeman (1934). The powder was suspended in 5 ml. 4 N-H_2SO_4, mixed with Gooch asbestos, and extracted with ether in a soxhlet for 24 hr. The ether extract was allowed to stand over anhydrous Na_2SO_4 overnight, filtered and evaporated to dryness. The residue was taken up in 10 ml. water and filtered. The filtrate was concentrated on a hot plate and then taken to dryness *in vacuo* over H_2SO_4 yielding at first colourless needles which on prolonged drying gave an opaque whitish product; yield 48·6 mg. of m.p. 179° (uncorr.) with decomposition. The melting point of anhydrous oxalic acid is 187° (corr.). The product was dissolved in ether, filtered, the filtrate was allowed to stand over anhydrous Na_2SO_4 overnight, filtered and evaporated to dryness. The residue redissolved in water and taken to dryness at 37° gave 46 mg. of colourless crystals, m.p. 100° (uncorr.). Mixed melting point with an authentic sample of hydrated oxalic acid (m.p. 101° uncorr.) was 100° (uncorr.). The yield based on glyoxylate disappearance was 58%.

L-*Lactic acid*

Tolbert *et al.* (1949) have shown that α-hydroxyacid oxidase catalyses the oxidation of L-lactic acid to pyruvic acid. D-Lactic acid is not attacked (Clagett *et al.* 1949). The effect of catalase on the oxidation of L-lactic acid catalysed by α-hydroxyacid oxidase is best shown with enzyme preparations freed from all but traces of catalase activity by treatment with calcium phosphate gel. With such preparations the addition of catalase almost halves the rate of O_2 uptake with lactate. The total O_2 uptake is 1 atom O/mol. L-lactic acid in presence of catalase but 1·6–1·8 atoms O/mol. L-lactic acid in absence of added catalase. Typical results are shown in Fig. 1. The composition of the reaction mixtures is given in Table 1. The results suggest that the reaction proceeds according to the equation

$$CH_3.CHOH.COOH + O_2 \rightarrow CH_3.CO.COOH + H_2O_2. \quad (5)$$

In the absence of catalase no H_2O_2 accumulates since on adding catalase when the O_2 uptake was complete no significant output was obtained. It has been shown by Negelein & Brömel (1939) with D-amino-acid oxidase using alanine as substrate that the H_2O_2 formed oxidizes the pyruvic acid, which is the other product of the enzyme-catalysed reaction, to acetic acid according to the equation

$$CH_3.CO.COOH + H_2O_2 \rightarrow CH_3.COOH + H_2O + CO_2. \quad (6)$$

Since pyruvic acid and H_2O_2 are the products of the action of α-hydroxyacid oxidase on L-lactic acid, the absence of accumulated H_2O_2 is presumably due to its reaction with pyruvic acid. This is supported by the values given in Table 1 for CO_2 output during the reaction and carbonyl compounds finally present in the reaction mixtures. In the absence of added catalase there was a high CO_2 output coupled with a low content of carbonyl compounds, and in the presence of added catalase a low CO_2 output coupled with a high content of carbonyl compounds. It is clear, therefore, that in the absence of catalase the net result of the oxidation of L-lactic acid by α-hydroxyacid oxidase is given by Eqns. (5) and (6), and in the presence of catalase by Eqns. (5) and (2).

DISCUSSION

The results of the present work show that the oxidation of L-lactic acid, glycollic acid, and glyoxylic acid catalysed by α-hydroxyacid oxidase takes place with the formation of H_2O_2. The final products of the oxidation depend on the amount of catalase present. Thus, according to Tolbert *et al.* (1949), pyruvic acid is the product of the oxidation of L-lactic acid. The present results suggest that this is only the case if sufficient catalase is present to stop the oxidation of the pyruvic acid to acetic acid by the H_2O_2 produced in the enzyme-catalysed reaction.

The oxidation of glycollic acid was shown by Tolbert *et al.* (1949) to proceed in two stages to formic acid and CO_2 with glyoxylic acid as the intermediate. According to the present results, formic acid is not the immediate product of the enzyme-catalysed reaction, but is formed by a secondary reaction between H_2O_2 and glyoxylic acid. In the complete absence of catalase the oxidation of glycollic acid should proceed entirely to

formic acid and CO_2, and that of glyoxylic acid to equimolecular amounts of formic acid and oxalic acid with evolution of CO_2 if, as seems likely, oxalic acid is the direct product of the enzyme-catalysed oxidation of glyoxylic acid. In the presence of sufficient catalase to stop the secondary reaction between glyoxylic acid and H_2O_2, no formic acid should be formed. As previously suggested, the most likely product under these circumstances would be oxalic acid. Nord & Vitucci (1947) have shown that certain fungi convert glycollic to oxalic acid with the postulated intermediate formation of glyoxylic acid.

In the present work the amounts of added catalase were such as to give a catalase activity of the same order as that of 3 ml. of tobacco-leaf sap. In view of the results obtained it appears that when untreated sap is used to catalyse the oxidation of lactate and glycollate its catalase activity would be sufficient to prevent the non-enzymic oxidation of pyruvate and glyoxylate by H_2O_2. Results with α-hydroxyacid oxidase preparations made from the sap would depend on the catalase activity of such preparations.

The influence of catalase on the oxidations catalysed by α-hydroxyacid oxidase provide an explanation of at least some of the variable O_2 uptakes and CO_2 outputs obtained by Clagett *et al.* (1949), Tolbert *et al.* (1949) and Tolbert & Burris (1950).

SUMMARY

1. Hydrogen peroxide is produced during the oxidation of lactate, glycollate and glyoxylate catalysed by plant α-hydroxyacid oxidase. This has been shown by the effect of catalase on the oxygen uptake.

2. The oxidation products depend on the amount of catalase present. In the absence of catalase, the enzyme-catalysed reactions are followed by non-enzymic reactions between hydrogen peroxide and pyruvate or glyoxylate, with the formation of acetate or formate respectively.

3. It is suggested that oxalate is the product of the enzyme-catalysed oxidation of glyoxylate.

REFERENCES

Agner, K. (1938). *Biochem. J.* **32**, 1702.
Clagett, C. O., Tolbert, N. E. & Burris, R. H. (1949). *J. biol. Chem.* **178**, 977.
Clift, F. P. & Cook, R. P. (1932). *Biochem. J.* **26**, 1788.
Dixon, M. (1943). *Manometric Methods*, 2nd ed. p. 64. Cambridge University Press.
Hatcher, W. H. & Holden, G. W. (1925). *Trans. roy. Soc. Can.* (3), **19**, 11.
Kenten, R. H. & Mann, P. J. G. (1952). *Biochem. J.* **52**, 125.
Kolesnikov, P. A. (1948*a*). *Dokl. Akad. Nauk. S.S.S.R.* **60**, 1205. Cited in *Chem. Abstr.* (1948), **42**, 7373*f*.
Kolesnikov, P. A. (1948*b*). *Biokhimiya*, **13**, 370. Cited in *Chem. Abstr.* (1948), **42**, 8889*b*.
Negelein, E. & Brömel, H. (1939). *Biochem. Z.* **300**, 225.
Nord, F. & Vitucci, J. C. (1947). *Arch. Biochem.* **14**, 229.
Pirie, N. W. (1946). *Biochem. J.* **40**, 100.
Pucher, G. W., Vickery, H. B. & Wakeman, A. J. (1934). *Industr. Engng Chem.* (Anal. ed.), **6**, 140.
Singer, T. P. & Kearney, E. B. (1950). *Arch. Biochem.* **29**, 190.
Sumner, J. B. & Somers, G. F. (1943). *Chemistry and Methods of Enzymes*, p. 171. New York: Academic Press Inc.
Tolbert, N. E. & Burris, R. H. (1950). *J. biol. Chem.* **186**, 791.
Tolbert, N. E., Clagett, C. O. & Burris, R. H. (1949). *J. biol. Chem.* **181**, 905.

The Biochemistry of the Nitrifying Organisms

1. THE AMMONIA-OXIDIZING SYSTEMS OF *NITROSOMONAS*

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Micro-organisms of the genus *Nitrosomonas* are autotrophs, typically found in soil, which oxidize ammonia to nitrite. Since the energy yielded by this oxidation is apparently the only primary energy source available to the organisms, no cell growth or proliferation can take place without ammonia oxidation. On the other hand, Nelson (1931) showed that ammonia oxidation could take place in the absence of carbon dioxide, i.e. under conditions that excluded the possibility of cell growth or proliferation, the energy released being (presumably) dissipated as heat instead of being used, in part at least, for carbon dioxide assimilation. It therefore follows that while any compound that inhibits ammonia oxidation by the organisms must inhibit their proliferation, the fact that a com-

pound inhibits proliferation cannot be taken as evidence that such a compound is inhibiting ammonia oxidation. A great deal of the confusion and contradiction that exists about the inhibitory effects of various compounds on 'nitrification by *Nitrosomonas*' can be traced to a failure to characterize the type of inhibition studied. The inhibitory actions of various compounds have, for instance, commonly been studied by means of experiments in which parallel inoculations of *Nitrosomonas* were made into two batches of culture medium, similar in all respects except that one batch contained some compound 'X' while the other did not. After some considerable period, usually days or weeks, the amount of nitrite formed in one culture was compared with the amount formed in the other. If the amount formed in the presence of 'X' was less than in its absence, 'X' was said to 'inhibit *Nitrosomonas*' or to 'inhibit nitrification by *Nitrosomonas*'. Clearly such experiments could give no indication how 'X' acted. If 'X' directly inhibited ammonia oxidation, then of course less nitrite would be formed in the presence of 'X' than in its absence. But if 'X' had no effect on ammonia oxidation and yet inhibited cell proliferation in some other way, the neogenesis of ammonia-oxidizing enzymes would be less rapid in the presence of 'X' than in its absence; as a consequence, nitrite formation in the presence of 'X' would be less than in its absence. In the present work the actions of various compounds on the formation of nitrite from ammonia by *Nitrosomonas* have been studied directly in experiments lasting not more than 3 hr.; since cell proliferation during this period must have been very small, it follows that inhibitions of nitrite formation noted under these conditions were predominantly due to an inhibition of ammonia oxidation. To the best of the author's knowledge, no such experiments have been performed before.

MATERIALS AND METHODS

Nitrosomonas was cultured from an Aberdeenshire garden soil (see below) by methods essentially similar to those already described (Lees, 1951). Minor modifications were, however, made in the design of the culture vessels and in the method of preparation of the inorganic culture medium.

Description of the organisms. The organisms so isolated, which were used throughout the present work, conformed precisely to the description of *N. europaea* given by Meiklejohn (1950). On silica gel, characteristic 'starfish colonies' (Meiklejohn, 1950) were obtained. It is therefore considered that the organisms were a normal strain of *N. europaea*.

Purity of the cultures. The commonly accepted criteria for the purity of *Nitrosomonas* cultures are (*a*) they should not form nitrate since the formation of nitrate from nitrite would indicate the presence of *Nitrobacter*, (*b*) they should show no growth on nutrient agar since growth on nutrient agar would indicate the presence of organisms other than *Nitrosomonas*. There is usually little difficulty in freeing the cultures of *Nitrobacter*, because *Nitrobacter* is inhibited by ammonia, which must, of course, always be present in growing cultures of *Nitrosomonas*. The important criterion of purity is therefore the lack of growth on nutrient agar; which, being a negative criterion, is of rather doubtful reliability (e.g. Nelson, 1931; Grace, 1951). The cultures used in the present work were not only pure by the two negative criteria mentioned, but had a high free-energy efficiency (Baas-Becking & Parks, 1927). The determinations of free-energy efficiency will form the subject of a future publication (Hofman & Lees, 1952). Since the presence of contaminants could have had no effect on the free-energy efficiency of the cultures except to lower it, it is considered that the high free-energy efficiency of the present cultures is a further indication of their purity.

Preparation of the medium. Although the medium originally described for the isolation of the organisms (Lees, 1951) proved satisfactory in most respects, it was necessary for the conduct of experiments on the free-energy efficiency of the cultures (Hofman & Lees, 1952) that the amount of adventitious organic material in the medium should be kept as low as possible. Since most laboratory chemicals seemed to be contaminated with specks of organic detritus, the medium used in the present work was prepared by precipitation from three solutions, each being filtered before use. Solution *A*: $CaCl_2$ (100 g.), $MgCl_2.6H_2O$ (100 g.), KH_2PO_4 (50 g.) dissolved in 1 l. of water slightly acidified with HCl to assist solution. Solution *B*: $FePO_4.2H_2O$ (1 g.) dissolved in 25 ml. N-H_2SO_4. Solution *C*: $Na_2CO_3.10H_2O$ (500 g.) dissolved in a minimum of warm water. Solutions *A* and *B* were mixed and added to solution *C*. The resulting precipitate of phosphates, sulphates, and carbonates of Ca, Mg, and Fe, was washed at the centrifuge with distilled water, until the washings were free of chloride, and finally suspended in 1 l. of distilled water. The medium on which the organisms were grown consisted of 10 ml. of this suspension, 1 ml. M-$(NH_4)_2SO_4$, and 89 ml. of distilled water. The principal difference in composition between this medium and the original medium (Lees, 1951) was that the new medium contained $MgCO_3$ while the original medium did not. The $MgCO_3$ raised the initial pH of the medium from 7·5 to 8·5, which is the optimal pH for nitrite formation by *Nitrosomonas* (Meyerhof, 1916).

Culture of the organisms

Cultures were grown in the dark at 28–30° in culture vessels whose design is shown in Fig. 1.

Sterilization of culture vessels. The 250 ml. glass jar containing 100 ml. of culture medium was plugged with cotton wool and sterilized in a small autoclave for 20 min. at 125°. Despite its alkalinity, the medium lost only a negligible amount of its NH_3 during this sterilization; the normal practice of sterilizing the $(NH_4)_2SO_4$ separately (e.g. Meiklejohn, 1950) would thus seem to be unnecessary. The rubber bung with its associated aerator and sampling assembly was not sterilized *in situ*, but was wrapped in paper, placed upside down in a beaker, and sterilized separately in the same autoclave. This procedure was used because when the bung was sterilized *in situ*, red dye from it sometimes seeped into the medium in the jar; *Nitrosomonas* would not grow in a medium so contaminated. After sterilization, the component parts of the culture vessel were assembled and inoculated with approximately 0·5 ml. of a culture of actively nitrifying *Nitrosomonas*, the vessel was

then attached to the air line through which air, sterilized by passage through two gas washers (Lees, 1949) (5% (w/v) $CuSO_4$ plus 5% (v/v) H_2SO_4), was supplied. From time to time, samples of approximately 2 ml. volume were withdrawn from the vessel via the sampler tube and analysed for NH_3-N, NO_2-N, and occasionally NO_3-N (see below). When the concentration of NH_3-N fell below 50 μg./ml. fresh NH_3 was added as sterilized $(NH_4)_2SO_4$ solution (if the pH of the sample, tested by B.D.H. (British Drug Houses Ltd.) Universal Indicator, was above 7·0) or as sterilized $(NH_4)_2CO_3$ (if the pH of the sample was below 7·0). It should be understood that the pH of the cultures must fall during growth of the organisms because of replacement of NH_4^+ by NO_2^-. The course of nitrite formation, commonly used as an indication of growth of *Nitrosomonas* cultures (Stephenson, 1949), in a typical culture used in the present work is shown in Fig. 2. Subcultures into fresh medium were made when the parent culture had formed 200–300 μg. NO_2-N/ml.

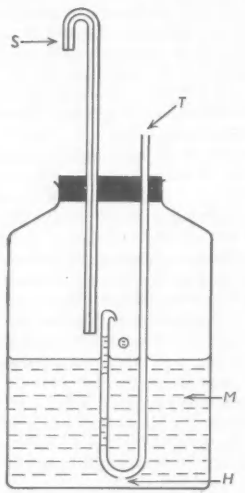

Fig. 1. Culture vessel. Sterile air is forced into the apparatus at *T*. In passing round the U-tube the air entrains bubbles of liquid medium *M* at the pin-hole *H*. The bubbles are carried by the air stream up the U-tube and expelled on to the surface of the liquid medium. The air escapes through the capillary tube *S*. The tube *S* can be dipped into the medium for the withdrawal of samples.

Incubation experiments

When the NO_2-N concentration of a growing culture of *Nitrosomonas* had risen above 200 μg. NO_2-N/ml., approximately 50 ml. of the culture were expelled through the sampler tube (*S*, Fig. 1) into a 100 ml. centrifuge tube, and centrifuged to bring down the precipitate of Ca and Mg carbonates to which the organisms adhere (Winogradsky, 1890*a*, *b*; Meiklejohn, 1950). The precipitate, with its adherent organisms, was washed two or three times at the centrifuge with water or with 0·9% NaCl solution to remove nitrite and NH_3, and finally resuspended in distilled water. Samples (2 ml.) of this suspension were incubated at 30° with solutions of the substance under test, both in the presence and absence of $(NH_4)_2SO_4$ solution, the final volume of suspension with all additions being 4 ml. Incubation was carried out in 50 ml. conical flasks, over the bottom of which the 4 ml. solution formed a thin, well aerated, layer. After the requisite period, the contents of the flasks were suitably diluted and centrifuged in conical centrifuge tubes to remove the inorganic carbonate precipitate. The NO_2-N in 5 ml. of the supernatant liquid was then determined (see below). In the present work, no nitrite was formed in the absence of NH_3, i.e. no compound tested, other than NH_3, gave rise to nitrite. No nitrite was formed if $(NH_4)_2SO_4$ solutions were incubated with boiled suspensions of *Nitrosomonas*. No appreciable amount of nitrite was formed if the incubation was carried out *in vacuo* or if the air in the flask was replaced by N_2. It therefore follows that the formation of nitrite under the experimental conditions used in this work was an aerobic, biological process, and that the nitrite formed was formed from NH_3.

Determination of NH_4-N. A 1 ml. sample of culture fluid was treated with approximately 2 ml. saturated borax

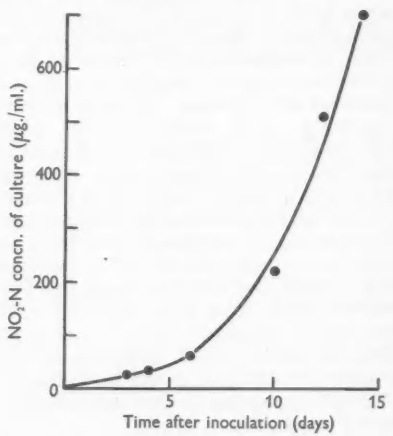

Fig. 2. The course of nitrite formation in a growing culture of *Nitrosomonas*. Culture volume, 100 ml.; inoculum volume, 0·5 ml.

solution and distilled from the Markham microdistillation apparatus (Markham, 1942) into 2 ml. 1% boric acid containing 0·25% of Conway and O'Malley's indicator (Conway & O'Malley, 1942). After about 5 ml. of distillate had been collected, the contents of the receiver were titrated with 0·007 N-HCl from a 1 ml. burette.

Determination of NO_2-N. A suitably diluted sample (5 ml.) of culture fluid (or fluid from an incubation experiment) was treated with 1 ml. of sulphanilic acid reagent followed by 1 ml. 1-naphthylamine reagent; the reagents were prepared according to Snell & Snell (1936). After 30 min. the light absorption of the treated sample was determined in an EEL colorimeter (Evans Electroselenium Ltd.) fitted with the 404 green filter supplied with the instrument. The NO_2-N content of the sample was obtained by reference to a standard curve constructed from the measured light absorptions of nitrite solutions of known strength, and

treated in the same way. The maximum amount of NO_2-N that could be used for a single estimation was about 2·5 μg., the minimum amount about 0·1 μg.; readings were repeatable to within 0·02 μg. within these limits.

Determination of NO_2-N. This was carried out according to the method used by Lees & Quastel (1946), except that the H_2O_2 oxidation was omitted. Under these conditions nitrite did not interfere with the estimation of nitrate by the phenoldisulphonic acid reagent (at least up to concentrations of 700 μg. NO_2-N/ml. sample).

Table 1. *Nitrite formations in washed suspensions of* Nitrosomonas *incubated for* 180 *min. in the presence of various compounds and* 10^{-3} M-$(NH_4)_2SO_4$

(All compounds were used at a concentration of 5×10^{-3} M except where otherwise stated. Final volume of washed suspension + additions = 4 ml.)

Compound tested	NO_2-N formed during incubation (μg.)
None (water control)	3·1
Glycine	3·2
L-Alanine	3·2
DL-Valine	3·1
L-Leucine	3·0
L-Isoleucine	3·0
L-Serine	3·0
L-Threonine	3·0
L-Aspartic acid	3·0
L-Glutamic acid	3·0
L-Lysine	3·0
L-Arginine	2·0
L-Histidine	0·0
L-Cystine ($2·5 \times 10^{-3}$ M)	3·0
DL-Methionine	3·0
L-Phenylalanine	2·5
L-Tyrosine	2·7
DL-Tryptophan	3·0
L-Proline	3·1
L-Hydroxyproline	3·0
L-Glutamine	3·0
L-Asparagine	3·0
DL-Methionine sulphoxide	2·6
Creatinine	2·6
Glycocyamine	2·6
2:4-Dinitrophenol	3·0
Ethylurethane	1·0
D-Mannose (5×10^{-2} M)	3·0
Streptomycin (Ca salt; 250 p.p.m.)	3·0

Soil percolation

Samples (10 g.) of garden soil were percolated at 28–30° according to the normal technique (Lees, 1949). The soil was a sandy loam from Milltimber, Aberdeenshire; pH, 6·8; organic matter, determined by the method of Bremner (1949), was 6%.

RESULTS

The effects of all the common amino-acids on the oxidation of ammonia by *Nitrosomonas*, together with the effects of various other compounds selected for reasons that will appear in the Discussion are shown in Table 1. The lack of any effect of streptomycin at a concentration of 250 p.p.m. is interesting, since streptomycin inhibits growth of the organisms at far lower concentrations (Fig. 3).

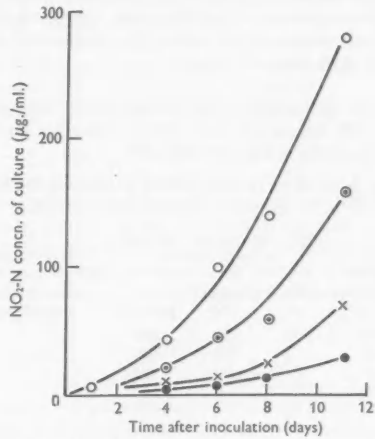

Fig. 3. The effect of streptomycin on nitrite formation in growing cultures of *Nitrosomonas*. Culture volumes, 100 ml.; volumes of inocula, 0·5 ml. (○—○), untreated medium; (●—●), streptomycin 0·4 p.p.m. in medium; (×—×), streptomycin 0·2 p.p.m. in medium; (⊙—⊙), streptomycin 0·1 p.p.m. in medium. Streptomycin was added to the medium as the Ca salt at the start of the experiments.

Table 2. *Nitrite formations in washed suspensions of* Nitrosomonas *incubated for* 90 *min. in the presence of various concentrations of* $(NH_4)_2SO_4$ *and a single concentration of inhibitor*

(Final volume of washed suspension + additions = 4 ml. Bracketed figures are nitrite formations expressed as percentages (to the nearest 10%) of the 'control' nitrite formation.)

$(NH_4)_2SO_4$ concn. (M)	NO_2-N (μg.) formed during incubation in presence of: No inhibitor (control)	L-Histidine $2·5 \times 10^{-4}$ M	Guanidine $1·2 \times 10^{-4}$ M	Allylthiourea 5×10^{-7} M
$2·5 \times 10^{-3}$	1·00	0·60 (60)	0·36 (40)	0·46 (50)
$1·2 \times 10^{-3}$	0·70	0·40 (60)	0·28 (40)	0·35 (50)
$6·2 \times 10^{-4}$	0·50	0·32 (60)	0·20 (40)	0·24 (50)
$3·1 \times 10^{-4}$	0·30	0·24 (80)	0·16 (50)	0·15 (50)
$1·6 \times 10^{-4}$	0·16	0·08 (50)	0·08 (50)	0·08 (50)
Nil	Nil	Nil	Nil	Nil

It has already been shown (Lees, 1946, 1948) that chelating agents inhibit nitrification in soil; the inhibitory effects of various chelating agents on ammonia oxidation by *Nitrosomonas* are shown in Fig. 4; histidine is included among the chelating agents since Maley & Mellor (1950) have shown that it is outstanding among the amino-acids in its power to bind metal ions. Further experiments with three of these inhibitors failed to reveal any evidence that the inhibitions produced were competitive with respect to ammonia, since the percentage inhibition of ammonia oxidation caused by one concentration of inhibitor was independent of ammonia concentration when this was varied over a fairly wide range (Table 2).

Table 3. *The breakdown of* DL-*methionine percolated as* 100 *ml. of approx.* 10^{-2} M *solution through* 10 *g. Aberdeenshire garden soil*

(The presence of volatile sulphur compounds in the air issuing from the percolator was detected by smell.)

Time from start of percolation (hr.)	Nitrogen (μg./ml.) present in percolate as NH_3-N (*a*)	Amino N (*b*)	(*a*) + (*b*)	Volatile sulphur compounds in air issuing from percolator
6	0	131	131	+
24	7	132	139	+ + +
48	27	97	124	+ +
96	55	80	135	–
144	77	60	137	–

Quastel & Scholefield (1949, 1951) have shown that low concentrations of DL-methionine inhibit soil nitrification as studied by a percolation technique. Percolation experiments carried out in this laboratory on the effect of percolated DL-methionine on soil nitrification have confirmed Quastel & Scholefield's finding, but, since the results in Table 1 afforded no indication that DL-methionine inhibited ammonia oxidation by washed suspensions of *Nitrosomonas*, it seemed possible that the inhibitions observed by Quastel & Scholefield, and confirmed here, might have been due to products formed from the percolated methionine by the soil heterotrophs. The breakdown of DL-methionine percolated through soil was therefore studied. The results of these experiments, given in Table 3, showed that deamination of percolated methionine was rapid. From an early stage in the percolation, the air issuing from the percolators had a foul smell reminiscent of hydrogen sulphide or mercaptans which indicated some breakdown of the methionine. Paper chromatograms carried out on samples of the percolate by Dr T. Hofman showed a gradual disappearance of the methionine, but no detectable production of methionine sulphoxide which Quastel & Scholefield (1951) suggested might be formed from percolated methionine. It thus seemed likely that the results of any experiments carried out with methionine under percolation conditions would be most difficult to interpret unequivocally and soil experiments on these lines were not continued.

DISCUSSION

Some of the compounds which the present results have shown to inhibit ammonia oxidation by *Nitrosomonas* have already been shown to inhibit either soil nitrification or the oxygen uptake of suspensions of *Nitrosomonas*. These compounds are guanidine (Meyerhof, 1916), allylthiourea, potassium ethyl xanthate, salicylaldoxime, and sodium diethyldithiocarbamate (Lees, 1946, 1948), and thiourea (Quastel & Scholefield, 1949, 1951). Inhibitions by L-histidine and 8-hydroxyquinoline have not, to the writer's knowledge, been previously reported. All these inhibitors can be subsumed under the heading 'chelating agents', and the simplest explanation of their effects is therefore that they inhibit the action of some metal enzyme. This is in agreement with the suggestion (Lees, 1946, 1948) that some metal enzyme is intimately concerned in ammonia oxidation by *Nitrosomonas*. Quastel & Scholefield (1951) have advanced an alternative theory to account for the inhibitory effects of guanidine, urethane, and thiourea; they suggest that 'It seems likely that the affinity of the N atom in the structure R.NH.C≡ for the ammonia-oxidizing enzyme is primarily involved.' There are, however, a number of objections to this theory. The inhibition of nitrite formation caused by 5×10^{-3} M-urethane in the present work was 65 % (Table 1); Quastel & Scholefield (1951) noted a 45 % inhibition of the oxygen uptake of a soil sample treated with ammonium chloride and 5×10^{-3} M-urethane, compared with the oxygen uptake in the absence of urethane. The agreement between these two results is good, but the degree of inhibition is of quite different order from the inhibition of nitrite oxidation brought about by chelating agents (Fig. 4), the weakest of which (L-histidine) was completely inhibitory at a concentration of $2{\cdot}5 \times 10^{-3}$ M. Moreover, as Quastel & Scholefield admit, arginine and creatine should, on their theory, inhibit ammonia oxidation by *Nitrosomonas*, whereas their own results failed to reveal any inhibition by these compounds. The 'affinity of the N atom in the structure R.NH.C≡' seems to imply competition between ammonia and R.NH.C≡ for some active site on the enzyme, yet inhibitions by guanidine and allylthiourea were apparently not competitive (Table 2); this finding is difficult to harmonize with Quastel & Scholefield's theory, but is consistent with the 'chelation' theory, which does not involve any concept of competition. In addition, the distribution of the points in Fig. 4 is what would be expected if all

the compounds used in determining the points had inhibited ammonia oxidation by a common chelation mechanism, since the slopes of the lines relating percentage inhibition with log. (inhibitor concentration) were all the same. Moreover, the weakest chelating agent, L-histidine, gave the weakest inhibition, while the stronger chelating agents gave correspondingly stronger inhibitions. There thus seem to be reasonable grounds for retaining the original 'chelation' theory, which has the merit of accounting for rather more facts.

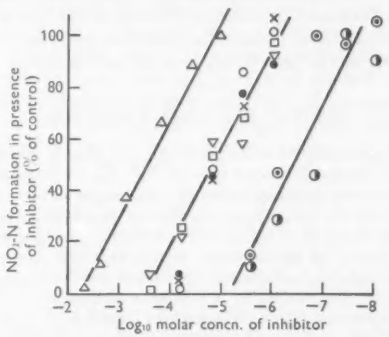

Fig. 4. The effects of various inhibitors on nitrite formation in washed suspensions of *Nitrosomonas* incubated for 180 min. in the presence of the inhibitor and 10^{-3} M-$(NH_4)_2SO_4$. Nitrite formations are given as percentages of the nitrite formation in similar washed suspensions, containing 10^{-3} M-$(NH_4)_2SO_4$, but without the addition of any inhibitor. (△—△), L-histidine; (▽—▽), guanidine; (●—●), 8-hydroxyquinoline; (□—□), salicylaldoxime; (○—○), potassium ethyl xanthate; (×—×), sodium diethyldithiocarbamate; (◑—◑), thiourea; (⊙—⊙), allylthiourea.

Jensen (1950) showed that D-mannose (6×10^{-3} M) inhibited growing cultures of *Nitrosomonas* studied over periods of several days, whereas the other common monosaccharides did not. The present work failed to reveal any direct effect of D-mannose within 3 hr. on nitrite formation by *Nitrosomonas* even at concentrations 8 times greater than those used by Jensen. This suggests that D-mannose inhibits some essential growth process in *Nitrosomonas* other than ammonia oxidation.

Despite the wide variety of nitrogenous compounds tested in the present work, none, except ammonia, was converted to nitrite by *Nitrosomonas*. This is in agreement with the generally accepted theory that ammonia is the obligatory primary oxidation substrate of *Nitrosomonas*. Although the stages by which the ammonia is converted to nitrite remain largely obscure there is now some evidence (Lees, in preparation) that hydroxylamine may be involved, as suggested by Kluyver & Donker (1926). If hydroxylamine, either free or as a bound hydroxamic acid (Kermack & Lees, 1952), is in fact formed during ammonia oxidation by *Nitrosomonas*, then the metabolism of *Nitrosomonas* would resemble in some respects the metabolism of *Azotobacter vinelandii*, where hydroxylamine appears to be involved both in the reduction of nitrate to ammonia and in ammonia oxidation (Virtanen & Jarvinen, 1951). The role of hydroxylamine in ammonia oxidation by *Nitrosomonas* is now being investigated in this laboratory.

SUMMARY

1. Nitrite formation by *Nitrosomonas* has been studied by means of short-term incubation experiments on washed suspensions of the organisms.

2. Despite the wide variety of nitrogenous compounds tested, only ammonia gave rise to nitrite under these conditions.

3. All chelating agents tested inhibited the formation of nitrite from ammonia by *Nitrosomonas*.

4. In a discussion of the results it is suggested that some metal enzyme is intimately concerned in ammonia oxidation by *Nitrosomonas*.

I am indebted to Prof. W. O. Kermack, F.R.S., for advice, help, and encouragement throughout this work. I also wish to thank Glaxo Laboratories Ltd., for a gift of streptomycin.

REFERENCES

Baas-Becking, L. G. M. & Parks, G. S. (1927). *Physiol. Rev.* **7**, 85.
Bremner, J. M. (1949). *Analyst*, **74**, 492.
Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.
Grace, J. B. (1951). *Nature, Lond.*, **168**, 117.
Hofman, T. & Lees, H. (1952). *Biochem. J.* **52**, 140.
Jensen, H. L. (1950). *Tidsskr. Planteavl*, **54**, 62.
Kermack, W. O. & Lees, H. (1952). *Sci. Progr.* **40**, 44.
Kluyver, A. J. & Donker, H. J. (1926). *Chem. Zelle*, **13**, 134.
Lees, H. (1946). *Nature, Lond.*, **158**, 97.
Lees, H. (1948). *Biochem. J.* **42**, 534.
Lees, H. (1949). *Plant & Soil*, **1**, 221.
Lees, H. (1951). *Nature, Lond.*, **167**, 355.
Lees, H. & Quastel, J. H. (1946). *Biochem. J.* **40**, 803.
Maley, L. E. & Mellor, D. P. (1950). *Nature, Lond.*, **165**, 453.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Meiklejohn, J. (1950). *J. gen. Microbiol.* **4**, 185.
Meyerhof, O. (1916). *Pflüg. Arch. ges. Physiol.* **166**, 240.
Nelson, D. H. (1931). *Zbl. Bakt.* (2. Abt.), **83**, 280.
Quastel, J. H. & Scholefield, P. G. (1949). *Nature, Lond.*, **164**, 1068.
Quastel, J. H. & Scholefield, P. G. (1951). *Bact. Rev.* **15**, 1.
Snell, F. D. & Snell, D. T. (1936). *Colorimetric Methods of Analysis*. London: Chapman and Hall.
Stephenson, M. (1949). *Bacterial Metabolism*. London: Longmans Green and Co.
Virtanen, A. I. & Jarvinen, H. (1951). *Acta chem. scand.* **5**, 220.
Winogradsky, S. (1890*a*). *Ann. Inst. Pasteur*, **4**, 257.
Winogradsky, S. (1890*b*). *Ann. Inst. Pasteur*, **4**, 760.

The Biochemistry of the Nitrifying Organisms

2. THE FREE-ENERGY EFFICIENCY OF *NITROSOMONAS*

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The free-energy efficiencies of a number of autotrophic micro-organisms have been calculated by Baas-Becking & Parks (1927) as the ratio between the free energy consumed in carbon dioxide assimilation and the free energy released by the primary inorganic oxidation. These calculations were based on the results of determinations, published by various workers, of the amounts of organic carbon found in initially inorganic media in which various autotrophs had oxidized measured amounts of substrates. In the case of *Nitrosomonas*, which obtains all the free energy needed for carbon dioxide assimilation from the free energy released by the oxidation of ammonia to nitrite, the calculation runs as follows: The reduction of 1 g. of carbon from carbon dioxide to the oxidation level at which carbon is found in cellular constituents requires approximately 10 000 cal. of free energy. The oxidation of 1 g. of nitrogen from ammonia to nitrite releases about 4 700 cal. of free energy. (The exact figure depends on the pH of the medium, concentrations of reactants, and other relevant factors.) The free energy efficiency of *Nitrosomonas* is therefore $\frac{C_o}{N_n} \times \frac{10\,000}{4\,700} \times 100\,\%$, where C_o = g. carbon assimilated by the culture and N_n = g. nitrite N formed by the culture. Baas-Becking & Parks (1927) used the value of 1/35, obtained by Winogradsky (1890*b*), for the ratio C_o/N_n and thus concluded that the free-energy efficiency of *Nitrosomonas* was about 6 %. This value has been widely accepted (e.g. Stephenson, 1949), although Nelson (1931) showed that it could be considerably exceeded, since analyses of his cultures of *Nitrosomonas* revealed that C_o/N_n was approximately 1/15, corresponding to a free-energy efficiency of 14 %. The results of Hes (1937) indicated that the free-energy efficiency of *Nitrosomonas* was somewhat higher in young cultures than in older ones, Winogradsky (1890*a*) had obtained similar indications in his preliminary investigations on carbon assimilation by *Nitrosomonas*, but he apparently regarded these preliminary results as unreliable. As Van Niel (1943) has pointed out, the whole subject of the free-energy efficiencies of the autotrophic micro-organisms might well be reinvestigated since the published data are too scanty to permit a reliable interpretation. The present work is a study of the free-energy efficiency of *Nitrosomonas* as defined above and determined at various stages during the growth of cultures of the organisms.

MATERIALS AND METHODS

The organisms were cultured in the way already described (Lees, 1952). For some experiments the volume of the cultures was increased to 400 ml. and a larger culture vessel used, but this change in no way affected the results obtained.

Determination of organic carbon formed in a culture. When the amount of nitrite formed in a culture had attained a suitable value, the whole of the culture was centrifuged to separate the precipitate of inorganic carbonates to which the organisms adhere (Winogradsky, 1890*a*, *b*; Meiklejohn, 1950). The precipitate, with its adherent organisms, was washed at the centrifuge with distilled water until the washings were free of NH_3, suspended in about 10 ml. of distilled water, and acidified with 10 N-H_2SO_4 to a pH of 2·0. The acid suspension was made up to a known volume and a measured sample transferred to a Van Slyke carbon tube (Van Slyke & Folch, 1940), together with 1 ml. conc. HCl to destroy any traces of carbonate. After the contents of the tube had been evaporated to dryness at 105°, the organic carbon in the residue was determined by the method of Van Slyke & Folch (1940). Blank determinations were made in a culture set up at the same time and in the same way as the 'test' culture but inoculated with a boiled inoculum.

Organic N *determinations.* From time to time the amount of organic N in a culture as well as organic C, was determined. For this determination, a sample of the suspension acidified with H_2SO_4 (see above) was digested according to a micro-Kjeldahl procedure and the final NH_3 determination carried out as already described (Lees, 1952). It was found that the ratio (organic C):(organic N) always fell between 3·5 and 4·0. The constancy of this ratio thus provided a check on the accuracy of the organic C determinations.

Nitrite N. This was determined as already described (Lees, 1952).

RESULTS

If the free-energy efficiency of *Nitrosomonas* is constant throughout growth of a culture $\frac{C}{N} = K$, where $C = \mu$g. organic C formed/ml. culture, $N = \mu$g. NO_2-N formed/ml. culture, K is a constant, therefore $\log C = \log K + \log N$, and a plot of $\log C$ against $\log N$ should yield a straight line of unit slope with an intercept equal to $\log K$ when $\log N$ is zero.

The experimentally determined points for log C and log N are shown in Fig. 1. The regression line shown, which was obtained by the least squares method, fits the equation $\log C = 0{\cdot}73 \log N - 0{\cdot}35$, whence $2{\cdot}2\,C = N^{0{\cdot}73}$. Thus $C/N = 1/(2{\cdot}2 \times N^{0{\cdot}27})$ and the free-energy efficiency (see Introduction) is $97/N^{0{\cdot}27}$ % or $77/C^{0{\cdot}37}$ %. The free-energy efficiency of *Nitrosomonas* thus falls as the NO_2-N and organic-carbon content of the culture increase. It should be noted that the free-energy efficiency so calculated is derived from the ratio of the total carbon assimilated to the total nitrogen oxidized; it may thus conveniently be thought of as an overall free-energy efficiency as contrasted with the instantaneous free-energy efficiency. A line calculated from the regression line and showing the fall of overall free-energy efficiency with increasing NO_2-N concentration is included in Fig. 1. Experimental points obtained by other workers are plotted for comparison.

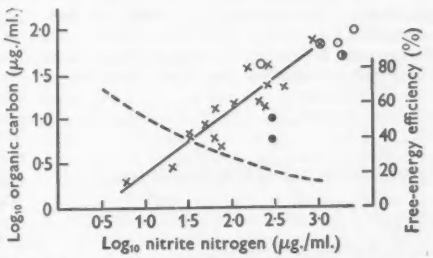

Fig. 1. The relation between organic carbon assimilated from carbon dioxide and nitrite nitrogen produced from ammonia in cultures of *Nitrosomonas*. Points determined by: (×), present authors; (○), Winogradsky, 1890*a*; (◑), Winogradsky, 1890*b*; (⊗), Nelson, 1931; (●), Bömeke, 1951. Solid line, regression line calculated to present results. Dotted line, free-energy efficiency calculated from regression line.

DISCUSSION

The results show that the overall free-energy efficiency of *Nitrosomonas* fell as the nitrite concentration and organic carbon concentration of the culture rose. The instantaneous free-energy efficiency of the organisms at any particular time is given by the first differential, dC/dN, of the expression relating the nitrite-nitrogen concentration with the organic-carbon concentration (see Results). It may therefore be easily calculated from the equation to the regression line that the instantaneous free-energy efficiency, dC/dN, is 0·73 of the overall free-energy efficiency.

The figure shows that the results obtained by other workers agree well with our own, with the exception of the results of Bömeke (1951) which indicate a rather lower amount of organic carbon in the cultures than might be expected from the amount of nitrite formed therein. Unfortunately the experimental basis of Bömeke's work was rather unsatisfactory. He did not actually measure the amount of nitrite formed in his cultures, but was content to assume that 80–90 % of the ammonia he added to the medium before sterilization was converted to nitrite. Moreover, he allowed his cultures to remain without ammonia, and in contact with the nitrite they had produced, for 3–4 weeks before performing any carbon analyses on them. His organisms were thus kept for a period in a state where respiration, with consequent loss of carbon, could proceed but where carbon assimilation, which is dependent upon ammonia oxidation, could not. It is therefore possible that there was an appreciable overall loss of carbon from the cultures during this 3- to 4-week period. Since Bömeke's 'nitrite' figures may be too high, and his carbon figures may be too low, there are reasonable grounds for supposing that the points corresponding to his analyses in Fig. 1 could, with advantage, be displaced upwards and to the left. This would bring them more closely into line with our own results and the results obtained by other workers. The observed fall in free-energy efficiency of growing cultures of *Nitrosomonas* may be due to either or both of two causes. If, as seems probable (Kermack & Lees, 1952), respiratory and oxidative activities in growing cultures of *Nitrosomonas* increase more rapidly than does the assimilative activity, then the free-energy efficiency of the cultures would fall during growth. Secondly, the end product of *Nitrosomonas* metabolism is nitrite, an ion highly toxic to most living organisms. It seems most unlikely that in view of this toxicity the intracellular nitrite concentration of *Nitrosomonas* could ever be high, certainly not as high as 0·1 M, a concentration often reached in *Nitrosomonas* cultures. Engel (1941) has suggested that ammonia oxidation by *Nitrosomonas* takes place on the cell surface; if this were so it may well be that nitrite never enters the cell at all and that the intracellular nitrite concentration is kept small. But since nitrite is not only toxic, but is easily diffusible, one is driven in any case to postulate that *Nitrosomonas* possesses some 'nitrite-excreting' mechanism for maintaining a concentration gradient of nitrite between the interior of the cell and the culture medium. Such a mechanism could not operate without a supply of free energy, and since the only primary source of free energy available to the organisms is ammonia oxidation, the operation of a 'nitrite-excreting' mechanism would demand the oxidation of some extra quantity of ammonia, over and above the quantity required to maintain the normal respiratory and assimilative requirements of the cell. Moreover, the rate at

which the nitrite-excreting mechanism consumed energy would increase as the nitrite concentration increased in the medium surrounding the cell. Hence, the free-energy efficiency of the cultures would fall with increasing nitrite concentration in the medium, i.e. it would fall as the cultures grew.

We now intend to investigate, by means of the Warburg technique, the respiration of *Nitrosomonas* cultures at various stages in their growth; we also hope to investigate the effect of various concentrations of nitrite on the respiration of the cultures. These experiments should lead to a better understanding of the causes underlying the fall in the free-energy efficiency of *Nitrosomonas* cultures during growth.

SUMMARY

1. The free-energy efficiency of *Nitrosomonas* falls as the amount of carbon assimilated and the amount of ammonia oxidized increase; the free-energy efficiency thus falls during the growth of cultures of the organisms.

2. The low free-energy efficiencies reported by other workers for *Nitrosomonas* are consistent with the high nitrite concentrations at which they were determined.

3. Possible reasons for the observed fall in free-energy efficiency are discussed.

We wish to acknowledge the help, advice, and encouragement of Prof. W. O. Kermack, F.R.S., throughout this work.

REFERENCES

Baas-Becking, L. G. M. & Parks, G. S. (1927). *Physiol. Rev.* **7**, 85.
Bömeke, H. (1951). *Arch. Mikrobiol.* **15**, 414.
Engel, H. (1941). *Forschungsdienst Sonderheft*, **16**, 144.
Hes, J. W. (1937). Thesis: University of Gröningen.
Kermack, W. O. & Lees, H. (1952). *Sci. Prog.* **40**, 44.
Lees, H. (1952). *Biochem. J.* **52**, 134.
Meiklejohn, J. (1950). *J. gen. Microbiol.* **4**, 185.
Nelson, D. H. (1931). *Zbl. Bakt.* (2. Abt.), **83**, 280.
Stephenson, M. (1949). *Bacterial Metabolism.* London: Longmans Green and Co.
Van Niel, C. B. (1943). *Physiol. Rev.* **23**, 338.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Winogradsky, S. (1890*a*). *Ann. Inst. Pasteur*, **4**, 257.
Winogradsky, S. (1890*b*). *Ann. Inst. Pasteur*, **4**, 760.

The Ultraviolet Absorption Spectrum of Ribonuclease

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No detailed study of the absorption spectrum of ribonuclease appears to have been made. It is well known that this enzyme contains no tryptophan, while its cystine and phenylalanine contents (Brand, 1948) are such as to make a negligible contribution to its absorption spectrum in the region above 2700 A. (see below). In addition, electrophoretic, ultracentrifugal and solubility studies all indicate homogeneity (Northrop, Kunitz & Herriott, 1948), although recent observations (Martin & Porter, 1951; Hirs, Stein & Moore, 1951) raise anew the question as to what is meant by a homogeneous protein preparation, at least in so far as ribonuclease is concerned.

The quantitative method introduced by Holiday (1936) for the spectrophotometric estimation of the tyrosine and tryptophan contents of proteins has been widely applied and has proved it is useful in a number of instances. There are, however, examples where serious divergences exist between the results obtained by this method, and chemical analyses. If, as appears most likely (Beaven, Holiday & Jope, 1950), absorption due to peptide linkages is negligible in the region of the absorption maxima of the aromatic amino-acids, it would seem reasonable to expect that a study of the differences obtained by the two methods may lead to information regarding the nature of the changes undergone by the aromatic amino-acids upon incorporation into a protein molecule. This assumption is naturally dependent upon the correctness of chemical analyses, regarding which doubts still exist in some instances (Tristram, 1950).

Further interest is lent to a study of ribonuclease by the observation of Kunitz (1940) that it is digested in acid medium by pepsin, with resulting denaturation and loss of enzymic activity. Since pepsin is known to attack linkages between α-carboxylic groups of dicarboxylic amino-acids and the α-amino radical of an aromatic amino-acid (Bergmann & Fruton, 1941), observation of the spectrum both before and after digestion may be

expected to throw some light on the origin of the fine structure spectral shifts observed for aromatic amino-acids in proteins (see Beaven *et al.* 1950, for recent review).

The absorption spectrum of this enzyme was reported by Uber & Ellis (1941), who found the maximum to be in the neighbourhood of 2800 A., with an extinction coefficient of $11{\cdot}4 \times 10^3$ at pH's 2·5 and 4·5, based on a molecular weight of 15000 and a nitrogen content of 16·1%. Their curve shows a minimum at about 2500 A. with a ratio of maximum to minimum extinction coefficient of 1·8.

EXPERIMENTAL

Two Kunitz preparations of ribonuclease (Kunitz, 1940) were kindly made available by Prof. J. Brachet. Cytological tests on one of these (Brachet & Shaver, 1948) indicated freedom from proteolytic activity, a fact confirmed by additional tests conducted during the course of an investigation of the ultraviolet inactivation of this enzyme (Shugar, 1951*a*). A few measurements were also made on a sample kindly supplied by Dr M. Macdonald and prepared according to her method (Macdonald, 1948). Tyrosine was an Eastman Kodak product, recrystallized from water; the nitrogen content agreed with that calculated from the dry weight and it showed only one component chromatographically. The pepsin used was a crystalline preparation from the Worthington laboratories. The lysozyme used in a comparison experiment was prepared according to the method of Alderton & Fevold (1946) and recrystallized four times.

All measurements were made in the 10 mm. cells of the Beckman spectrophotometer (two instruments were used) generally at 10–20 A. intervals except in the neighbourhood of maxima, minima and points of inflexion, where 5 A. intervals were used. The blank cell always contained all the other constituents of the enzyme solution. Special precautions were taken with respect to cell corrections which were measured over the entire spectral range with distilled water in the cells. In order to obtain measurements which could be duplicated to within 1%, it was found necessary to ensure that the same cell face be struck by the incident light beam. Where constituents were added to solutions in the cells following one series of measurements, dilution corrections were made. Enzyme concentrations were so adjusted that most of the readings fell in the range of optical densities from 0·3 to 0·8.

Buffers were made up with double-distilled water and checked with a Beckman pH meter using glass electrodes; 0·1 N-HCl was taken as pH 1, 0·01 N-HCl as pH 2, 0·1 N-NaOH as pH 13. Sørensen phosphate buffers were used in the range 5·4 to 8·7 and 0·1 M-glycocoll buffers above this range.

Results are given as extinction coefficients $\epsilon = d/c$, where d is the optical density for a 10 mm. path length and c the concentration in moles/l.

RESULTS

Curve *A* of Fig. 1 shows the absorption spectrum of ribonuclease at pH 7·2; the principal maximum is at 2775 A. with an extinction coefficient of $11{\cdot}4 \times 10^3$, based on a molecular weight of 15000 (Northrop *et al.* 1948) and a nitrogen content of 16·5% (Brand, 1948). The striking resemblance of the spectrum to that of tyrosine is obvious (Fig. 4, curve for pH 7·2). Curve *B* of Fig. 1 is the spectrum in 0·1 N-NaOH, the maximum being at 2930 A., which is also the position of the maximum of free tyrosine at the same pH (Fig. 4). However, this is not the form of the spectrum obtained immediately upon bringing the enzyme solution to pH 13; the absorption at first increases rapidly, to about 75% of the height of curve *B* during the first 2 min., then

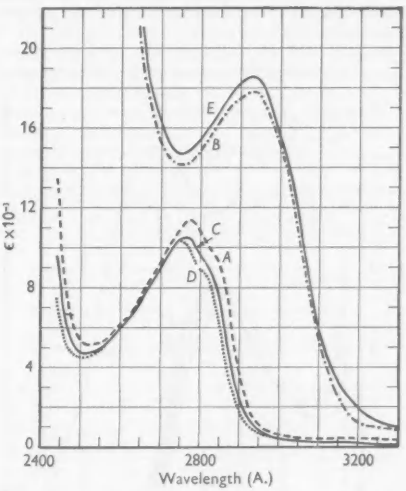

Fig. 1. Absorption spectrum of ribonuclease: *A*, in phosphate buffer, pH 7·2; *B*, in 0·1 N-NaOH; *C*, in 0·1 N-HCl; *D*, following digestion by pepsin in 0·1 N-HCl; *E*, in 0·1 N-NaOH, following digestion by pepsin.

rises slowly to its final value. In one typical experiment where the optical density at 3000 A. attained a final value of 0·439, the optical densities following addition of alkali were, at 2, 3, 4, 5, 6, 7, 9, 13, 19 and 36 min. respectively 0·331, 0·351, 0·375, 0·390, 0·400, 0·409, 0·418, 0·429, 0·439 and 0·439. Warming the solution at this point resulted in no further change. As will be shown below, what is being observed here is the alkaline denaturation of the enzyme. The change in absorption in going from neutral to alkaline solution is due to the dissociation of the phenolic groups of tyrosine (Crammer & Neuberger, 1943) and the partial time-dependence of this change would appear to indicate that at least 25%, and probably more, of these groups in the ribonuclease molecule are not free to ionize at all until denaturation has taken place.

Curve *C* of Fig. 1 shows the spectrum of ribonuclease in 0·1 N-HCl. The absorption maximum has undergone a hypsochromic shift of 10 A. while the extinction coefficient has been reduced 8%. At this point, 20 cu.mm. of a solution of pepsin (2 mg./ml.) were added to the enzyme solution which was then left overnight, since no marked change was immediately obvious. Curve *D* represents the spectrum following the digestion period. It will be seen that the principal maximum is now at 2750 A., which is that for

tyrosine itself; and that the second maximum of tyrosine (Fromageot & Schnek, 1950) noticeable in the original curve *A*, has become more pronounced and is now close to 2800 as in tyrosine, Fig. 4. The ratio of the two maxima in the undigested ribonuclease is 1·13, in the digested enzyme it is 1·16, while for tyrosine itself it is 1·18 (Fig. 4 and Fromageot & Schnek, 1950).

Following digestion, the enzyme solution was brought to pH 13, resulting in curve *E* of Fig. 1. As in the case of the undigested solution it was necessary to wait more than 15 min. for the curve to reach its final form, indicating that, even following digestion, some of the phenolic groups are still not free to ionize.

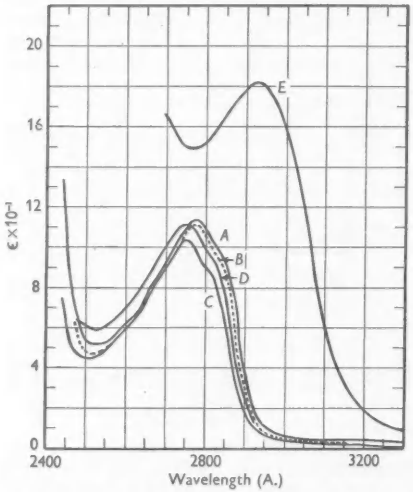

Fig. 2. Absorption spectrum of ribonuclease: *A*, in phosphate buffer, pH 7·2; *B*, in 0·01 N-HCl; *C*, following digestion by pepsin in 0·01 N-HCl; *D*, at pH 7·2, following digestion; *E*, in 0·1 N-NaOH following digestion.

Fig. 2 illustrates another typical experiment in which the digestion was carried out at pH 2. Curve *A* is the original spectrum; curve *B* is that in 0·01 N-HCl, only slightly different from that in neutral solution. Curve *C* is that following digestion and is identical with that obtained at pH 1. However, the rate of digestion was sufficiently rapid at pH 2 to follow the course of the reaction approximately. One hour following addition of pepsin (temperature about 25°) the absorption maximum had shifted to 2765 A. with very little change in extinction coefficient; after another hour the maximum was between 2755 and 2760 A., while the extinction coefficient had dropped 5%. Several hours more were required to reach curve *C*.

Curve *D* resulted when the digested protein was brought to pH 7 with NaOH. While the maximum has remained at 2750 A., the extinction coefficient is now somewhat higher than in the original solution; it did not, however, remain stationary but dropped slowly towards curve *A*, indicating the presence of secondary reactions. Further addition of alkali to bring the pH to 13 resulted in curve *E*, almost identical with that of Fig. 1; in this instance 85% of the height of the curve was attained within 1 min., suggesting that some of the 'bound' phenolic groups had been liberated as a result of digestion. It was still necessary to wait almost 15 min. for curve *E* to be attained.

A plot of the spectrum of the enzyme at various pH values results in the series of curves shown in Fig. 3. It will be seen that up to pH 12·3, all the curves pass through two isosbestic points at 2700 and 2800 A., denoting the presence of a reversible ionic equilibrium due to the dissociation of the phenolic groups of tyrosine. No sooner, however, is pH 12·3 exceeded than the resulting curves break away from the isosbestic points and, in fact, begin to increase in height at a rate which increases with pH. The curve for pH 12·3 itself passes through the isosbestic points only because it was

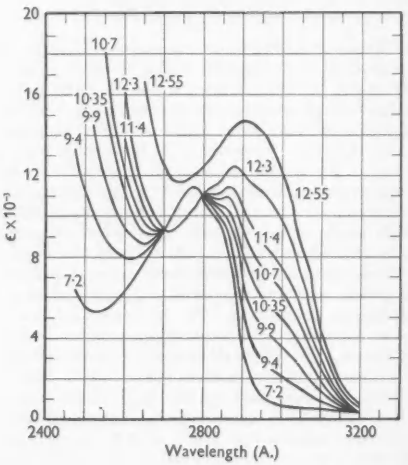

Fig. 3. Absorption spectrum of ribonuclease at pH values indicated. Note isosbestic points at 2700 and 2800 A. Between these two wavelengths, at pH values between 9·4 and 11·4, the curves have not been drawn in, for the sake of clarity.

recorded rapidly; within another half-hour it had already changed slightly. Below pH 12·3, the spectra remained unaltered for periods of some hours at room temperature. It is clear that above pH 12 denaturation takes place; that this latter process is irreversible was demonstrated by the fact that neutralization of the solution resulted in a new spectrum, the maximum of which was quite broad, 2740–2770 A., with an extinction 20% higher than that of the original neutral solution; the minimum was now at 2610 A. while the absorption above 3000 A. was appreciable.

The ionic equilibrium indicated by Fig. 3, if due to the ionization of the phenolic groups of tyrosine, should be shown by tyrosine itself. Fig. 4 shows the absorption spectrum of tyrosine at several pH values. It will be seen that there is one clearly defined isosbestic point at 2670 A., and another at 2775 A., except for the curve at pH 7·2 which does not pass through it. Although the deviation of the pH 7·2 curve from this point is small (about 4%), repeated trials showed that it is real and that, not until pH 9 had been passed, did all the curves intersect at this second

isosbestic point. While the reason for this was at first ascribed to some possible impurity in the tyrosine, it should be recalled that the α-amino group of this amino-acid has a pK of about 9 (Haurowitz, 1950) so that the dissociation of this group may be expected to have a small effect on the spectrum in the neighbourhood of pH 9, even though the phenolic ring is separated from the α-amino radical by two intervening single bonds (Braude, 1949). In the ribonuclease molecule itself, the amino group of tyrosine is bound in a peptide linkage and so this effect would be absent, as it is (Fig. 3).

A comparison of Figs. 3 and 4 shows that the isosbestic points for tyrosine undergo a bathochromic shift of 30 and 25 A. respectively, when this amino-acid is incorporated into the ribonuclease molecule, which corresponds quite well to the 25 A. shift undergone by the maximum of the tyrosine curve.

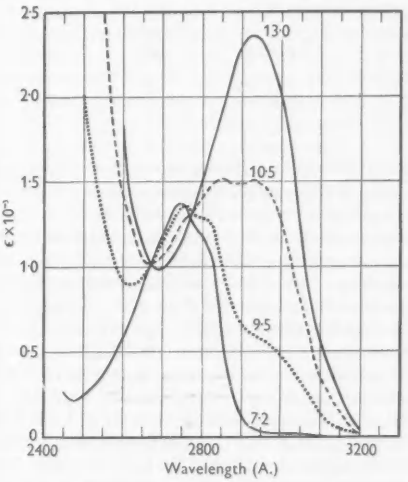

Fig. 4. Absorption spectrum of tyrosine at pH values indicated. Note isosbestic points at 2670 and 2775 A. See text for discussion of isosbestic point at 2775 A.

From the curves in Fig. 4 it is readily shown that the alkaline pK of tyrosine is 10·0 as compared with the values of 10·05 obtained by Crammer & Neuberger (1943) and 10·1 by Fromageot & Schnek (1950).

A plot of the increase in the absorption of ribonuclease at a given wavelength, between 2600 and 3050 A., as a function of pH, yields the solid curve of Fig. 5, from which the apparent pK for dissociation of the phenolic groups is 11·4, if we include also those groups which dissociate only upon denaturation. For purposes of comparison, the analogous results of Crammer & Neuberger (1943) for insulin have been plotted to the same scale and are shown as the dotted line in the same figure. It will be seen that both curves are similar up to pH 12; above pH 12, insulin still bears some resemblance to a normal 'titration' curve (see, however, below), while that for ribonuclease deviates sharply from this behaviour. That the insulin curve represents an equilibrium reversible even at pH 13 is shown by an examination of Fig. 1 of the paper by Crammer & Neuberger (1943); extrapolation of several of their curves shows that, up to and including pH 13, all of them pass through two isosbestic points at about 2700 and 2800 A. Only above pH 13 do the spectra break away from these points.

Applying a correction for the presence of tryptophan, Fromageot & Schnek (1950) showed that the ionization of the tyrosyl phenolic groups of lysozyme follows a completely normal dissociation curve with an apparent pK of 10·8 and that the spectrum was entirely reversible up to pH 13. Elsewhere it has been shown that lysozyme exposed to alkali and brought back to neutral pH suffers no loss in activity (Shugar, 1951*b*). Some of the spectral measurements of the above authors have now been repeated with substantially the same results; in particular, bringing a lysozyme solution directly to pH 13 results in the same curve reported by them, with no time lag perceptible in its attainment, in agreement with their findings, and indicating that the phenolic groups of lysozyme are not partially 'bound' as in ribonuclease.

Some of the principal numerical constants for tyrosine and ribonuclease are listed in Table 1. The values for tyrosine at pH 1 are in good agreement with those of Fromageot &

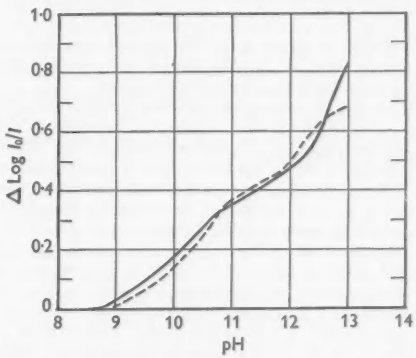

Fig. 5. Relative increase in optical density of ribonuclease at 3000 A. as a function of pH (solid line). The dotted line has been drawn from the observations of Crammer & Neuberger (1943) for insulin, and adjusted to the same scale for purposes of comparison.

Schnek (1950) as are those at pH 13. The latter are also in good agreement with a mean value based on the measurements of a number of observers (Holiday, 1951). However, the extinction coefficient for pH 7·2 is in marked disagreement with that of Fromageot & Schnek (1950), $1{\cdot}23 \times 10^3$ and with that of Holiday (1936), $1{\cdot}24 \times 10^3$. By contrast both Sizer & Peacock (1947) and Kretchmer & Taylor (1950) find no difference in extinction coefficients between pH 1 and 8, as compared to the slight difference found here, and the 11% difference reported by Holiday (1936) and Fromageot & Schnek (1950). The reason for these discrepancies at neutral pH is not clear.

The figures cited for ribonuclease are for the Kunitz preparation proved to be free from proteolytic activity, and based on nitrogen determinations made on solutions measured spectrophotometrically; one determination of

extinction coefficient was also made from the dry weight, the enzyme being dried at 110° for more than 12 hr. as suggested by Brand & Kassell (1942), and indicating a moisture content of 15%. The value found for the extinction coefficient is quite close to that reported by Uber & Ellis (1941); but the ratio of maximum to minimum extinction coefficients is 2·2 as compared to the value of 1·8 reported by the latter authors. The preparation supplied by Dr Macdonald gave an almost exactly similar spectrum, but with a slightly higher extinction coefficient and a ratio of maximum to minimum absorption of 2·3. The importance of this ratio has already been pointed out (Shugar, 1951*b*) and will be referred to again in the Discussion (see below).

Application of the method of Holiday (1936) to the calculation of the tyrosine and tryptophan contents of ribonuclease from the curve in alkaline solution in Fig. 1 yields (*a*) tryptophan = 0, in agreement with chemical analyses and (*b*) tyrosine = 10 residues/mol. ribonuclease as compared to the 6·6 residues determined chemically by Kunitz (see Northrop *et al.* 1948) and Brand (1948).

If, however, the tyrosine content is calculated more directly from the ratio of the extinction coefficients of ribonuclease in acid solution following digestion and that for tyrosine at pH 1, the result is 7·6 residues/mol. ribonuclease. A similar calculation for the two curves at pH 13 gives 7·5 residues. Even the mean of these two values is 13% higher than the chemically determined one, suggesting the presence of absorption due to other sources than that of free tyrosine.

Table 1. *Principal absorption bands, extinction coefficients and isosbestic points for tyrosine and ribonuclease*

	Absorption maxima			Isosbestic points		
Substance	pH	λ (A.)	ε (×10⁻³)	pH range	λ (A.)	ε (×10⁻³)
Tyrosine	1·0	2750	1·36	9·5–13·0	2670	1·03
	7·2	2750	1·38	9·5–13·0	2775	1·33
	13·0	2930	2·36	—	—	—
Ribonuclease	1·0	2765	10·5	7·0–12·3	2700	9·25
	2·0	2775	11·1	7·0–12·3	2800	11·0
	7·2	2775	11·4	—	—	—
	13·0	2930	17·8	—	—	—
Digested ribonuclease	1·0–2·0	2750	10·4	—	—	—

DISCUSSION

A comparison of the curves for tyrosine and ribonuclease, including the relative heights of their isosbestic points and the minima at about 2500 A., indicates the presence of absorption in the enzyme other than that of free tyrosine, such extraneous absorption increasing with decreasing wavelength. If we accept Brand's (1948) analysis for the cysteine, cystine and phenylalanine contents of ribonuclease, and the extinction coefficients of Fromageot & Schnek (1950) for these amino-acids, their combined absorption accounts for only 15% of that of the enzyme at 2500 and 2600 A., 2·5% at 2700 A. and practically zero at 2800 A. and above. It is quite conceivable that the binding of 40% of the phenolic groups may result in some change in absorption; the fact, however, that calculations in both acid and alkaline media (in the latter of which all the phenolic groups are liberated) leads to the same tyrosine content, argues against this being the cause of the higher absorption.

In view of the low molecular weight of ribonuclease, absorption due to scattered light may be neglected. There remains the possibility of some secondary effects related to the peptide linkages. As already pointed out above, available evidence appears to exclude direct absorption by peptide linkages in this region of the spectrum. This does not, however, exclude the possibility that a non-aromatic amino-acid, bound in a peptide linkage to an aromatic amino-acid, may influence the absorption of the latter, notwithstanding that the aromatic ring is separated from the linkage by two single bonds (Braude, 1949). A secondary effect of this nature has already been postulated to account for the small wavelength shifts observed in peptides containing an aromatic amino-acid (Beaven *et al.* 1950). An examination of the spectra of Fromageot & Schnek (1950) for leucyltyrosine and tyrosylcysteine does indeed show increases in extinction, above that of tyrosine, of the order of 10–20% at 2750 A. at pH 8 (at pH 13 the difference is smaller); while at 2500 A. that of tyrosylcysteine is about three times as high as for tyrosine, so that the ratio of its maximum to minimum absorption is about 2·2, almost exactly that for ribonuclease in neutral solution (Fig. 1), although too much significance should not be attached to this concordance. It is, however, not without interest to note that this ratio is not markedly altered for ribonuclease following digestion, as would be expected from the above facts if only the linkage at the α-amino group is hydrolysed by pepsin. The decrease in extinction coefficient at 2500 A. accompanying digestion is of the same order as that found for horse serum globulin (Haurowitz & Astrup, 1939) and serum albumin and globulin (Beaven *et al.* 1950).

While the above facts readily account for the 13% difference between the chemically and spectroscopically determined tyrosine contents, it should not be overlooked that the amino-acid analysis of

ribonuclease leads to a calculated molecular weight somewhat higher than that determined by physical methods (Northrop *et al.* 1948). If the amino-acid analysis of Brand (1948) is really correct for the sample he examined, the question arises as to whether different methods of preparation do indeed result in exactly the same final product. As pointed out in the Results section, a second Kunitz preparation and one prepared by Dr M. Macdonald gave results differing by a few per cent from those listed in Table 1. The increasing precision becoming available for both amino-acid analyses and spectrophotometric measurements make it obviously desirable to remove these doubts by using the same preparation for both series of measurements (see also below).

In a recent communication, Rideal & Roberts (1951) postulate the existence of some absorption by peptide bonds at 2500 A. They find (as is the case for ribonuclease, above) that insulin, bovine serum albumin and gelatin display somewhat higher absorption at this wavelength than that of solutions containing the equivalent concentrations of free amino-acids, and conclude that about 5% of the absorption at this wavelength is due to the peptide bonds. In view of what has been said above, it appears somewhat difficult to accept such a conclusion without further evidence. These authors assume that gelatin contains no tyrosine, whereas in fact gelatin is known to contain small but varying proportions of tyrosine which have been measured both chemically and spectrophotometrically (Beaven *et al.* 1950). As further evidence they cite the fact that the absorption of acetylglycine at 2537 A. is higher than that of glycine, and conclude that, since the absorption of the methyl group is small, the increased absorption must be due to absorption by the peptide linkage; such a conclusion is not in agreement with the widely differing results reported by Fromageot & Schnek (1950) for leucyltyrosyl and tyrosylcysteine, and is at variance with the observation of Edwards (1949) that acetyltryptophan has a lower absorption than that of free tryptophan. Finally, their curve for insulin shows a ratio of maximum to minimum absorption of 2·0, as compared with 2·5 for that of Crammer & Neuberger (1943) and 1·8 for a curve presented by Beaven *et al.* (1950); the differences in absorption between these three curves at 2500 A. are of the same order of magnitude as the difference found by Rideal & Roberts (1951) between their preparation and that of an equivalent solution of free amino-acids.

Very few reliable measurements exist with regard to the changes resulting from peptide binding on the absorption spectra of di- and tri-peptides of aromatic amino-acids, except in so far as the fine structure of these spectra is concerned (Beaven *et al.* 1950). It is, however, clear from the above that further data of this nature are required adequately to explain the differences observed between the absorption spectra of proteins and their constituent amino-acids.

The reversion of the position of the entire absorption band of the enzyme to the position of that for tyrosine, as a result of digestion, is also readily explicable on the basis of the influence of the adjacent bound amino-acids. That it is not the peptide linkage itself which causes the shift is shown by the data of Beaven *et al.* (1950) where the extent of the shift varies with the nature of the amino-acid bound by the aromatic amino-acid. Although they report shifts of the order of only 5–15 A. as a result of peptide combination (compared to the 25 A. shift which occurs in ribonuclease), their measurements were made on peptides, the non-aromatic amino-acids of which were only glycine, cystine or alanine. The mere fact that the shifts caused by these three amino-acids are different, suggests that others may produce more (or less) appreciable shifts. The fact, however, that acidification to pH 1 causes a 10 A. shift in ribonuclease (leaving only 15 A. to be accounted for by digestion, in accord with Beaven *et al.*) does suggest that part of the displacement of the spectrum may be due to reasons other than peptide binding of tyrosine. Tyrosine shows no such shift in acid (Table 1); nor does insulin, although its absorption spectrum is displaced 20–30 A. from that of tyrosine (Crammer & Neuberger, 1943).

The few experiments which have been reported on the effect of digestion upon the displacement of the absorption bands of proteins have generally been conducted on molecules containing major proportions of more than one aromatic amino-acid. Further studies could much more profitably be made upon proteins containing only one main absorbing constituent such as gramicidin (tryptophan only), zein or insulin (tyrosine only). Insulin is of special interest because it is digested by both pepsin and chymotrypsin, each of which attacks different types of bonds; its amino-acid composition is fairly well established (Brand, 1946; Fromageot, 1950); while considerable studies have been made on the digestion products (Sanger & Tuppy, 1951; Stephen, 1951).

If we omit the 40% of the phenolic groups in ribonuclease which dissociate only upon denaturation, the apparent pK of the remaining groups is 10·4. (This value is obtained from Figs. 4 or 5, graphically or by calculation equally well at 2600 as between 2800 and 3100 A.) Crammer & Neuberger (1943) obtained a pK of about 11 for the phenolic groups in insulin (actually 10·8 from the curve in Fig. 5 of this paper, based on their measurements) and ascribed this higher value (as compared to 10·05 for free tyrosine) to the increasing net charge

of the molecule with increasing pH. It should, however, be noted that, from titrimetric measurements the phenolic pK of leucyltyrosine is 10·2; that of tyrosylcysteine 10·5; glycyltyrosine 10·4 and tyrosylarginine 9·6 (Cohn & Edsall, 1943). The values for leucyltyrosine and tyrosylcysteine have been confirmed spectrophotometrically by Fromageot & Schnek (1950). It is not at all unlikely that, in tripeptides containing the tyrosine in the middle, the variation of the phenolic pK from that of free tyrosine may be even more accentuated, so that the phenolic pK values observed in proteins may be related principally to the nature of the amino-acids to which the tyrosine residues are directly bound. In so far as ribonuclease is concerned, the pK value of 10·4 for the 'free' phenolic groups can hardly be considered anomalous. An examination of the data of Table 5 of the paper by Crammer & Neuberger (1943) for insulin suggests, indeed, the presence of at least two sets of phenol groups, one of which dissociates much more readily than the other, as would be expected from the above considerations if the tyrosine residues were linked to more than one other type of amino-acid. The same result may be deduced from the form of the dotted curve of Fig. 5 of this paper.

With regard to those phenolic groups not free to dissociate prior to denaturation, the situation is somewhat analogous to that for egg albumin, where very few of these groups are able to ionize below pH 13 (Crammer & Neuberger, 1943), a result confirmed titrimetrically by Cannan, Kibrick & Palmer (1941). It is difficult to visualize an explanation for this phenomenon other than that advanced by Crammer & Neuberger (1943), namely, some type of hydrogen bonding of the phenolic groups. However, their suggestion that it is these bonds which tend to endow the native protein with a certain degree of stability is in contradiction with the observed facts for ribonuclease which has fewer 'bound' phenolic groups, but which is so much more resistant to heat denaturation than is egg albumin.

Crammer & Neuberger also report (1943) that denaturation of egg albumin, as measured spectrophotometrically, is instantaneous at pH 13. While this may very well be true, they did not employ a spectrophotometer and were obliged to use a spectrographic technique which is not as readily amenable to the rapid study of spectral changes. In so far as ribonuclease is concerned, it appears that the alkaline denaturation (or inactivation) may be followed quantitatively by the above procedure.

The finding that digestion at pH 2 does liberate some of the 'bound' phenolic groups is similar to the situation prevailing for bovine serum albumin (Sizer & Peacock, 1947), a protein which also contains an appreciable percentage of 'bound' phenolic groups. Further confirmation for the existence of these bound groups is forthcoming from the work of Sizer (1946, 1947), who showed that the tyrosyl groups of insulin are more readily attacked by tyrosinase than those of serum albumin; more recent studies (Haas, Sizer & Loofbourow, 1951) suggest some modification of this interpretation.

The results of Tanford (1950) on human serum albumin are rather curious. Spectrophotometric measurements show an apparent pK for the phenolic OH groups of 11·7, with no signs of dissociation below pH 10. From titrimetric data, the phenolic pK is calculated to be 9·6. Tanford (1950) discounts the validity of his spectrophotometric results because of the presence of tryptophan. However, the tryptophan content of serum albumin is very small compared to its tyrosine content (Haurowitz, 1950), and its change with pH is much smaller than that of tyrosine at wavelengths in the neighbourhood of 3000 A., so that the spectrophotometric results look much more convincing than is suggested by the author, and suggest the need for a re-examination of the titrimetric results.

SUMMARY

1. The ultraviolet absorption spectrum of ribonuclease has been studied over the pH range 1–13.

2. The tyrosine content calculated from the spectral data corresponds to 7·5 residues per molecule ribonuclease.

3. The dissociation of the phenolic groups of tyrosine in ribonuclease is shown to correspond to the dissociation of the same groups in tyrosine; 40 % of these groups in the ribonuclease molecule are bound and can ionize only after denaturation of the molecule; the apparent pK of the remaining 60 % is 10·4.

4. Digestion of the enzyme with pepsin accentuates the resemblance of the ribonuclease spectrum to that of tyrosine.

5. The differences between the ribonuclease and tyrosine spectra are accounted for qualitatively on the basis of secondary effects resulting from peptide linkages. The nature of these effects is discussed.

REFERENCES

Alderton, G. & Fevold, H. L. (1946). *J. biol. Chem.* **164**, 1.
Beaven, G. H., Holiday, E. R. & Jope, E. M. (1950). *Discuss. Faraday Soc.* no. 9, 406.
Bergmann, M. & Fruton, J. S. (1941). *Advanc. Enzymol.* **1**, 63.
Brachet, J. & Shaver, J. R. (1948). *Stain Tech.* **23**, 177.
Brand, E. (1946). *Ann. N.Y. Acad. Sci.* **47**, 187.
Brand, E. (1948). See Northrop, Kunitz & Herriott (1948).
Brand, E. & Kassell, B. (1942). *J. biol. Chem.* **145**, 365.
Braude, E. A. (1949). *J. chem. Soc.* p. 1902.
Cannan, R. K., Kibrick, A. C. & Palmer, A. H. (1941). *Ann. N.Y. Acad. Sci.* **41**, 243.
Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides.* New York: Reinhold Publishing Co.
Crammer, J. L. & Neuberger, A. (1943). *Biochem. J.* **37**, 302.
Edwards, B. G. (1949). *Arch. Biochem.* **21**, 103.
Fromageot, C. (1950). *Cold Spr. Harb. Sym. quant. Biol.* **14**, 49.
Fromageot, C. & Schnek, G. (1950). *Biochim. Biophys. Acta*, **6**, 113.
Haas, W. J., Sizer, I. W. & Loofbourow, J. R. (1951). *Biochim. Biophys. Acta*, **6**, 589.
Haurowitz, F. (1950). *Chemistry and Biology of Proteins.* New York: Academic Press.
Haurowitz, F. & Astrup, T. (1939). *Nature, Lond.*, **143**, 118.
Hirs, C. W., Stein, W. H. & Moore, S. (1951). *J. Amer. chem. Soc.* **72**, 1893.
Holiday, E. R. (1936). *Biochem. J.* **30**, 1795.
Holiday, E. R. (1951). Personal communication.
Kretchmer, N. & Taylor, R. (1950). *J. Amer. chem. Soc.* **72**, 3291.
Kunitz, S. (1940). *J. gen. Physiol.* **24**, 15.
Macdonald, M. (1948). *J. gen. Physiol.* **32**, 39.
Martin, A. J. P. & Porter, R. R. (1951). *Biochem. J.* **49**, 215.
Northrop, J. H., Kunitz, S. & Herriott, R. M. (1948). *Crystalline Enzymes*, 2nd. ed. New York: Columbia University Press.
Rideal, E. K. & Roberts, R. (1951). *Proc. roy. Soc.* A, **205**, 391.
Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463.
Shugar, D. (1951*a*). In preparation.
Shugar, D. (1951*b*). *Biochim. Biophys. Acta* (in the Press).
Sizer, I. W. (1946). *J. biol. Chem.* **163**, 145.
Sizer, I. W. (1947). *J. biol. Chem.* **169**, 303.
Sizer, I. W. & Peacock, A. C. (1947). *J. biol. Chem.* **171**, 767.
Stephen, Joan M. L. (1951). *Biochem. J.* **49**, 629.
Tanford, C. (1950). *J. Amer. chem. Soc.* **72**, 441.
Tristram, G. R. (1950). *Chem. & Ind.* pp. S 868–S 873.
Uber, F. M. & Ellis, V. R. (1941). *J. biol. Chem.* **141**, 229.

Further Observations on the Preparation and Composition of the Hyaluronic Acid Complex of Ox Synovial Fluid

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Ogston & Stanier (1950) concluded that hyaluronic acid occurs in synovial fluid in combination with protein. The undegraded state of this complex in solution, prepared by ultrafiltration, was judged by viscosity measured in an Ostwald viscometer, and by sedimentation in the ultracentrifuge. It was not possible to account satisfactorily for the composition of the complex in terms of the analytical values for its components.

The main object of the present work was to make better and more extensive analyses. As a preliminary, methods of preparation were examined in the hope of finding one which would give undegraded material more conveniently than by ultrafiltration. In this search, a more sensitive test of degradation, based on the variation of viscosity with velocity gradient, was used. It is concluded that ultrafiltration remains the method of choice and improved analyses of material thus prepared are reported.

The results of this work generally confirm the previous conclusions, though altering some of them in detail. Nearly the whole weight of complex is now accounted for in terms of protein, acetylglucosamine, glucuronic acid and mineral content.

In the course of the work, the zero-concentration value of the sedimentation constant of the complex has been re-determined and the action of hyaluronidase has been briefly investigated. The values given by Ogston & Stanier (1951*a*) for the physical dimensions of the particle of complex have been adjusted in the light of the new results.

METHODS

The methods for collection and storage of synovial fluid, preparation of the mucin clot from 1 ml. of fluid and determination of dry weight described by Ogston & Stanier (1950) were used.

The ultrafilter residue. This was prepared by the method described by Ogston & Stanier (1950), using a sintered-glas

'5 on 3' filter of average pore diameter about 1 mμ. (made by Baird and Tatlock Ltd.). 100–200 ml. of synovial fluid were used on each filter and the loss of hyaluronic acid through the filter was small.

Glucosamine. This was estimated by the method of Johnston, Ogston & Stanier (1951).

Glucuronic acid. This was estimated by the method of Ogston & Stanier (1951*b*). It has been found that protein makes a very small contribution to the CO_2 evolved during the glucuronic acid determination (1 mg. protein ≡ 0·023 ml. 0·01 N-HCl) and the results have been corrected accordingly.

Total nitrogen. This was estimated by micro-Kjeldahl, using selenium catalyst and 7–8 hr. digestion; a Markham (1942) apparatus was used for distillation.

Ash. This was determined on samples of hyaluronic acid complex, first dried to constant weight at 105° and then ignited at 550°.

pH. This was determined using an electrode of Corning 015 glass and an AgCl reference electrode in 0·1 M-KCl, with a liquid junction, phthalate and borate buffers (Cecil, 1951) being used to calibrate.

Ions. Na and Ca were determined by flame photometry (Leyton, 1951) in material which had been ignited at 550–600°, dissolved in HCl and centrifuged.

Paper chromatography. For amino-acids this was carried out by the methods of Williams & Kirby (1948) and Consden, Gordon & Martin (1944). For carbohydrates irrigation with 40 % *n*-butanol-10 % ethanol-50 % water (v/v) on Whatman no. 1 filter paper and development with aniline hydrogen phthalate was employed.

Ultraviolet absorption. This was measured in 0·1 N-NaOH using a Beckman spectrophotometer or a Hilger 'Uvispek' spectrophotometer.

Refractive increment. This was measured in the refractometer described by Cecil & Ogston (1951).

Viscosity. This was measured at 25° either in an Ostwald or Couette viscometer, as described by Ogston & Stanier (1950).

Ultracentrifuge. A Svedberg oil-turbine ultracentrifuge with a Philpot (1938) optical system was used. The procedure of running, and methods of calculating sedimentation constants and estimating the quantities of components were as described by Cecil & Ogston (1948).

Electrophoresis. A Hilger Tiselius-type apparatus with a Philpot (1938) optical system was used.

Standard buffer. This contained 0·2 M-NaCl, 0·0077 M-Na_2HPO_4 and 0·0023 M-KH_2PO_4 (pH 7·3), and was used for all measurements of refractive increment, viscosity, sedimentation and electrophoresis.

RESULTS

Anomalous viscosity as a criterion of degradation

Ogston & Stanier (1951*a*) showed that the variation of viscosity with velocity gradient and concentration can be represented by a plot of

$$\log\{(\eta_{rel.}-1)/\phi\}$$

against ϕ, where ϕ is the concentration expressed as volume fraction of unsolvated hyaluronic acid complex (Fig. 1). This method of plotting was suggested by Dr J. P. Johnston. An index of the degree of variation of viscosity with velocity gradient is given by the difference Δ between the values of $\log\{(\eta_{rel.}-1)/\phi\}$ extrapolated to zero and to infinite velocity gradient at a stated value of ϕ. Table 1 shows: (i) that values of Δ are reasonably constant for native fluids, (ii) that a marked fall of Δ of derived material may occur without a corresponding change of viscosity measured in an Ostwald viscometer or of intrinsic viscosity ($\phi=0$). Δ thus appears to be a more sensitive test of degradation than those previously used. Fig. 1 shows examples of the behaviour of an undegraded (*a*) and a degraded (*b*) material.

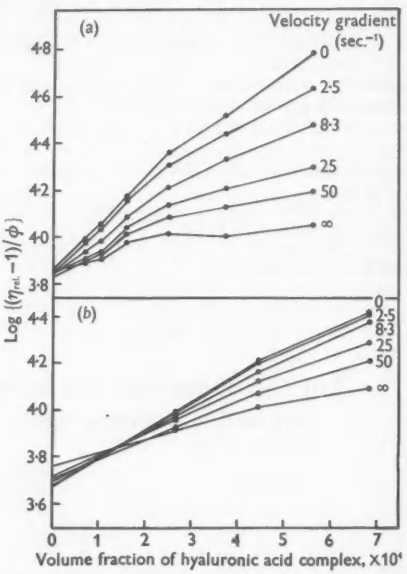

Fig. 1. Variation of $\log\{(\eta_{rel.}-1)/\phi\}$ with volume fraction ϕ at different velocity gradients. (*a*) synovial fluid; (*b*) hyaluronic acid complex precipitated by ethanol.

Methods of preparation

Mucin. Ogston & Stanier (1950) found that a single precipitation of hyaluronic acid as mucin might not greatly affect the Ostwald viscosity. It now appears (Table 1) that even a single precipitation produces a large lowering of Δ. Therefore no method of preparation which involves precipitation of mucin is satisfactory.

Precipitation with ethanol. Ropes, Robertson, Rossmeisl, Peabody & Bauer (1947) used precipitation with ethanol in alkaline solution at −20° to prepare hyaluronic acid free of protein after a preliminary precipitation of mucin; Ogston & Stanier (1950) concluded that this method would give an undegraded product, provided that the protein content was not reduced too much. Since

the mucin precipitation is undesirable, we have now used a preliminary concentration of synovial fluid, and partial removal of protein, on a glass filter; this starting material showed a value of Δ equal to that of the original fluid. Precipitation with ethanol at $-20°$ was then carried out, both on the material maintain good hyaluronic acid boundaries in a small electrophoresis cell, though their behaviour in the 150 ml. cell was better. Experiments were carried out because of the claim by Blix (1940) to have separated from synovial mucin by this method hyaluronic acid containing only 10 % of protein.

Table 1. *Values of* $\log\{(\eta_{rel.}-1)/\phi\}$ *for all products*

(All measurements at 25° except Couette measurements on fluid 29*K* (19°.)

			$\phi = 7\times10^{-4}$ ml./ml.				$\phi = 0$
			Couette: velocity gradient G sec.$^{-1}$				Couette: (all velocity gradients)
Material	No.	Treatment	0	∞	Δ	Ostwald	
Fluid	29*K*	—	4·67	3·87	0·80	3·74	3·94
	36*A*	—	4·97	4·21	0·76	3·85	3·89
	37*A*	—	4·97	4·06	0·91	3·90	4·00
	53	—	4·65	3·92	0·73	—	—
	54	—	4·86	4·00	0·86	—	—
	55	—	4·80	3·99	0·81	—	—
Mucin	26*K*	One pptn.	4·09	3·69	0·40	—	3·65
	28*A*	One pptn.	3·97	3·66	0·31	3·41	3·55
	35*K*	One pptn.	4·21	3·88	0·33	3·73	3·54
Hyaluronic acid complex ppted. by ethanol	38–40	Three pptn. from twice ppted. mucin	4·49	4·10	0·39	4·02	3·72
	50	One pptn. from alkaline conc. fluid	4·67	4·08	0·59	3·92	3·99
	51	One pptn. from neutral conc. fluid	4·34	3·83	0·51	3·60	3·65
Hyaluronic acid complex prepared by ultrafiltration	33	32 washings: no toluene	4·36	3·96	0·40	3·85	—
	35*K*	55 washings: no toluene	4·42	4·03	0·39	3·87	3·74
	55	Toluene: three washings	4·75	3·95	0·80	—	—
		Eight washings	4·87	3·98	0·89	—	—
		16 washings	4·88	4·03	0·85	—	—
		28 washings	4·76	4·03	0·73	—	—
		39 washings	4·57	4·01	0·56	—	—

dissolved in water, and after making it alkaline with NaOH as in the method of Ropes *et al.* (1947). The protein content was reduced by both procedures, though more so with alkali. Both products had a normal Ostwald viscosity, but a reduced Δ, showing some degree of degradation, though not so much as is produced by precipitation as mucin (Table 1). Ethanol precipitation after precipitation of mucin causes no further lowering of Δ (Table 1). The lowering of Δ is not caused merely by the removal of free protein in this method, since adding synovial fluid protein to a sample made by ethanol precipitation caused no increase in the value of Δ. It seems, therefore, that the process of precipitation with ethanol causes degradation.

Electrophoresis. The use of electrophoresis as a preparative method was not considered seriously, since it seemed likely to be more trouble and to give lower yields than ultrafiltration. The high viscosity of solutions of hyaluronic acid made it impossible to

A sample of mucin made on the larger scale (Ogston & Stanier, 1950) was run in the 10 ml. cell. The boundaries due to free protein were measured, and their contribution to the total refractive increment was compared with that of total protein, taken as 78 % of mucin. The free protein was less by 12 % than the total protein, and this means that 25 % of the hyaluronic acid complex is protein. The amount of albumin was estimated from the photographs to be 80–93 % of the total free protein. In the ultracentrifuge (Ogston & Stanier, 1950) only one free component was visible, sedimenting at the rate of serum albumin.

A second experiment was carried out in the 150 ml. cell with synovial fluid which had been concentrated on a glass filter. After running for a suitable time, four samples were removed by pipette from different levels of the upper section and two from the lower section of the anode limb. These were analysed for total nitrogen and glucosamine (Table 2). The

lowest value for the ratio of total nitrogen to glucosamine indicates a higher proportion of protein than is found in the best ultrafilter products (Table 3). All the samples showed normal Ostwald viscosity.

Table 2. *Composition of fractions taken from anode side of Tiselius apparatus*

	Fraction	Total N (mg./ml.)	Glucosamine (mg./ml.)	Total N/glucosamine
Top section	1	0·113	0·226	0·50
	2	0·055	0·123	0·45
	3	0·077	0·210	0·37
	4	0·045	0·159	0·28
Second section	5	0·596	0·225	2·65
	6	0·704	0·216	3·26

Ultrafiltration. Although it had been shown by the earlier criteria that ultrafiltration produces undegraded material, it was now necessary to find out whether this material has the full degree of anomalous viscosity possessed by synovial fluid. Two samples, which had been judged to be undegraded

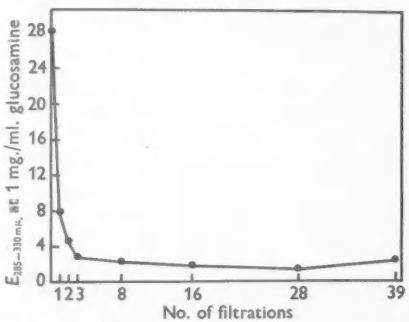

Fig. 2. Course of removal of free protein from synovial fluid by successive filtrations and washings.

by the earlier criteria, were found to have a lowered value of Δ (Table 1). This question was therefore investigated. Pooled fluid 55 was washed as usual, except that the saline and water used in washing were saturated with toluene. Samples taken at different stages of washing were dialysed and their viscosities were measured in the Couette viscometer. Their concentrations were estimated after dialysis on the basis of their glucosamine contents, these being converted to volume fraction of hyaluronic acid (see below). The results (Table 1) show that there was no degradation after 16 filtrations, possibly slight degradation after 28 filtrations and definite degradation after 39.

An approximate measure of the removal of the free protein by filtration was obtained from the values for the differences in extinction measured at 285 and at 330 mμ. (Fig. 2). This showed that the first three filtrations had removed some 90 % of the total protein of synovial fluid. Fig. 2 and the values of the ratio of total N to glucosamine (Table 3) show that further filtration removes protein at a very slow rate. However, a rise of the N/glucosamine ratio has occurred after 39 filtrations.

It seems, therefore, that ultrafiltration brings the protein content down to a fairly stable low value and that between 16 and 28 filtrations can be performed without any sign of degradation appearing by any of the criteria available.

Composition of the hyaluronic acid complex made by ultrafiltration

The material prepared by 16 filtrations (samples 16*W* and 16*D*) was subjected to more complete analysis, with a view to resolving the discrepancies noted by Ogston & Stanier (1950). Dialysed samples made by 28 and 39 filtrations (28*D* and 39*D*) were less completely analysed (Table 4).

Sample 16*W* was in solution in water; it was centrifuged at 5000 *g* to remove a small amount of solid matter. 16*D* was made by dialysing a sample of 16*W* against buffer. During this dialysis, a further small amount of solid separated and was removed by centrifuging. The N/glucosamine and dry weight/glucosamine ratios (Table 4) of 16*D* are less than those of 16*W*. These changes would be accounted for if the solid which separates on dialysis were protein. This was found to be the case when the N and glucosamine contents of another sample of hyaluronic acid complex were measured before and after dialysing against buffer and centrifuging: in this case, the N content showed a similar lowering while the glucosamine content was unchanged.

The molar ratio of glucuronic acid to glucosamine of 16*W* is 1·34. This ratio was determined on several other samples (Table 5), the ratio being in all cases well above 1·0. The ash content of 16*W* agreed well with a determination on another sample.

Although the composition of sample 28*D* (dialysed and centrifuged) differs little from that of

Table 3. *Progress of removal of protein N by ultrafiltration*

Sample	3*D*	8*D*	16*D*	28*D*	39*D*
No. of filtrations	3	8	16	28	39
Glucosamine (mg./ml.)	0·255	0·248	0·256	0·282 ·	0·249
Total N (mg./ml.)	0·086	0·077	0·067	0·065	0·076
Total N/glucosamine	0·34	0·31	0·26	0·23	0·30
Mucin (mg./ml.)	2·26	2·26	—	—	—

Table 4. *Analysis of hyaluronic acid complex made by ultrafiltration*

Sample	16*W*	16*D*	28*D*	39*D*
Dry wt. (mg./ml.)	1·28	1·09*	1·11*	1·04*
Glucosamine (mg./ml.)	0·280	0·256	0·282	0·249
Glucuronic acid (mg./ml.)	0·405	—	—	—
Ash (mg./ml.)	0·077	—	—	—
Na^+ (μequiv./ml.)	0·70	—	—	—
Ca^{++} (μequiv./ml.)	0·24	—	—	—
pH	6·1	—	—	—
Refractive increment $\times 10^5$	19·2	16·4	16·6	15·6
Total N (mg./ml.)	0·090	0·067	0·065	0·076
Protein N (mg./ml.) (Total N – glucosamine N)	0·068	0·047	0·043	0·057
Protein (mg./ml.) (protein N × 6·45)	0·44	0·30	0·28	0·37
Hyaluronic acid (mg./ml.) (dry wt.-protein)	0·84	0·79	0·83	0·67
Total N/glucosamine	0·32	0·26	0·23	0·30
Dry wt./glucosamine	4·6	4·2	3·9	4·2
Glucosamine % of hyaluronic acid	33	32	34	37
Protein % of dry wt.	34	28	25	36

* From refractive increment.

Table 5. *Glucuronic acid : glucosamine ratios in hyaluronic acid complex produced by ethanol precipitation and by ultrafiltration*

Sample	Protein (%)	Glucosamine (%)	Glucuronic acid (%)	Molar ratio glucuronic acid : glucosamine
One precipitation from ethanol	69	5·5	7·2	1·20
Several precipitations from ethanol	18	26	35	1·24
Ultrafiltration residue	34	23	32	1·28
Ultrafiltration residue (16*W*)	34	22	32	1·34

16*D*, sample 39*D* shows distinct changes of the N/glucosamine ratio and N content on a dry weight basis (Table 4).

Table 6. *Composition of hyaluronic acid complex*

Component	g./100 g. of complex
Glucosamine	(24·8)
Acetylglucosamine	30·6
Glucuronic acid	35·9
Protein	25·7
Ash	6·8
	99·0
Less 1 mole water/mole glucosamine	2·5
Less 1 mole water/mole glucuronic acid	3·3
	93·2

Since all the physical measurements reported by Ogston & Stanier (1950, 1951*a*) were made on dialysed and centrifuged material, it is the composition of this material (i.e. of 16*D* rather than 16*W*) which is of chief interest. However, since the dry weight determined directly is probably more accurate than that determined from the refractive increment, and since only the latter is available for dialysed samples, the composition on a dry weight basis given in Table 6 is for 16*W*, after subtraction of the weight of protein (assumed 15·5% N) removed following dialysis. This gives the most accurate estimate of the composition of dialysed material.

Composition of mucin

Mucin clots were made from 1 ml. of solution of ultrafilter residue to which synovial fluid protein had been added, by the method used for synovial fluid. Their dry weights were determined in duplicate and agreed within 2%. It was confirmed that further washing of the clots produced no loss of weight.

According to these results (Table 3), glucosamine forms 11·2% of mucin; from this and from the proportion of glucosamine in hyaluronic acid complex (Table 6), hyaluronic acid complex is found to form 45% of mucin. These values are higher than those given by Ogston & Stanier (1950), which were based on analysis of mucin made on a larger scale. Since Ogston & Stanier (1951*a*) assumed that hyaluronic acid complex forms only 32% of mucin (based on their previous work) their values for the physical constants of the complex require emendation (see below).

Chromatographic analysis

A two-dimensional chromatographic analysis for amino-acids was performed on a sample of hyaluronic acid and, in a parallel experiment, on a sample of serum albumin. No marked difference

between the chromatograms could be observed. This suggests that the protein in the complex has the usual N content (15·5 %).

Attempts were made to detect the presence of any carbohydrate component other than glucosamine and glucuronic acid, following treatment with aqueous or methanolic hydrochloric acid, and paper chromatography; the results of these experiments were entirely negative.

Treatment with hyaluronidase

A few experiments were made on the action of a crude preparation of rat-testis hyaluronidase, in the hope that this might lead to a method for isolating the protein part of the hyaluronic acid complex following depolymerization of the hyaluronic acid. The action of the enzyme was followed by measuring the viscosity of a portion of the enzyme-containing solution, which contained about 1 mg./ml. of hyaluronic acid. The relative viscosity fell from 20·6 to 1·75 after 24 hr. and to 1·51 after 48 hr. At these times samples were examined in the ultracentrifuge. In each case a single diffuse boundary was observed. These boundaries had sedimentation constants 2·07 and $1{\cdot}97 \times 10^{-13}$ (confirming Hahn, 1947) and represented respectively 83 and 58 % of the original refracting material. Since the protein in the original samples represented only 30 % of the refracting material, it is clear that the boundaries must still have contained a large amount of carbohydrate material, and that the hyaluronic acid was only partially depolymerized (see Discussion). The attempt to separate the protein in this way was abandoned.

During the treatment with hyaluronidase, the separation of a small amount of a granular precipitate was noticed. Treatment of a sample of hyaluronic acid complex with Benger's 'Hyalase' also led to the precipitation of about 7 % of the weight of dissolved material. This caused no detectable change of the N/glucosamine ratio. The 'Hyalase' was found to be without detectable proteolytic activity.

Sedimentation of purified hyaluronic acid complex

The values of the sedimentation constant at zero concentration and of its variation with concentration used by Ogston & Stanier (1951*a*) were obtained with dilutions of synovial fluid dialysed against buffer. Dr J. P. Johnston (personal communication) has observed that free protein may modify the rate of sedimentation of the complex. The opportunity was, therefore, taken to study the sedimentation of sample 16*D* over a wide range of concentration (Fig. 3). No sign of free protein could be observed. The boundary of hyaluronic acid remained as sharp as in synovial fluid at the same concentrations. The rates of sedimentation at lower concentrations were close to those observed with synovial fluid; at higher concentrations, the sedimentation constants in synovial fluid were somewhat lower (Fig. 3) and this agrees with the findings of Johnston.

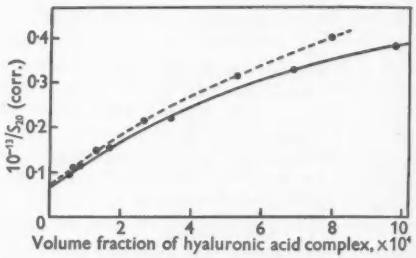

Fig. 3. Variation of sedimentation constant with volume fraction of hyaluronic acid complex. - - -●- - -, synovial fluid (Ogston & Stanier, 1950); —●—, ultrafiltration residue 16*D*.

DISCUSSION

The use of the variation of viscosity with velocity gradient as a criterion has confirmed the conclusion of Ogston & Stanier (1950) that ultrafiltration does not lead to degradation of the hyaluronic acid complex, whereas its precipitation by acid (as mucin) or by ethanol does cause degradation. The persistent presence of a proportion of protein in the complex is confirmed, though the amount of this protein is less than was estimated by Ogston & Stanier (1950). This difference was due to their analysing the solution in water, produced by ultrafiltration, whereas on dialysis against buffer a constant fraction of the protein separates as a precipitate. It is likely that this is denatured protein held in suspension, which separates when the viscosity is lowered by buffer. We have found that electrophoresis does not remove more protein than does ultrafiltration, whether the starting material is synovial fluid or mucin; this is contrary to the finding of Blix (1940).

Composition of the hyaluronic acid complex. Of the weight of the complex 93 % can now be accounted for in terms of acetylglucosamine, glucuronic acid, protein and ash (Table 6). The following points deserve mention: (i) the molar ratio of glucuronic acid to glucosamine in the complex is markedly greater than the value of 1·0 claimed by Meyer, Smyth & Dawson (1939); (ii) it is assumed that the carbohydrate components are joined by anhydride links, so that the weight of 1 mole of water has been subtracted for each mole of glucosamine and of glucuronic acid; (iii) the assumption that the protein contains near to 15·5 % N is confirmed by chromatographic comparison of its amino-acid composition with serum albumin; (iv) the complex yields some 7 % of ash: Na^+ and

Ca^{++} together account for 1·8 % of the dry weight or 0·83 m-equiv./g. dry weight compared with 1·85 m-equiv. of glucuronic acid.

The change of composition which is associated with the onset of degradation observed in sample 39*D* is not in line with the observation of Ogston & Stanier (1950), who concluded that lowered N content is associated with degradation. Here the proportion of N to glucosamine, and of N to dry weight, have increased. Such changes could be due to microbial action.

Ionic composition and pH. Comparison of the amount of metallic ions with that of carboxyl groups suggests that less than half of the carboxyl groups are ionized. Since the pK of glucuronic acid is about 3·5 (using the value found by Karrer & Schwarzenbach, 1934, for galacturonic acid), and since the pH of the solution is 6·1, nearly complete ionization might be expected. However, Katchalsky (1951) has shown that the pH of a solution of half-ionized polymethacrylic acid may be several units higher than the pK of the monomer, owing to the interaction of the charged groups. In accordance with the observations of Katchalsky & Spitnik (1947) on polymethacrylic acid, the pH of a solution of hyaluronic acid complex was found to fall markedly when neutral salt was added.

The composition of mucin. This is not in itself of any importance since no such complex appears to exist in synovial fluid and since its separation is not a desirable stage in the preparation of undegraded hyaluronic acid. Nevertheless, the estimation of mucin is a useful method for measuring the amount of hyaluronic acid in the presence of protein and has been much used for this purpose. It is important to realize, therefore, that its composition may depend on the method of preparation. Small clots made on glass rods and thoroughly washed appear to have a constant composition: this differs from the composition of mucin made on a larger scale, probably through the presence in the latter of entrained protein.

The action of hyaluronidase. Treatment, even for a long period, leaves a considerable proportion of the hyaluronic acid in a state in which its sedimentation is observable in the ultracentrifuge. Hahn (1947) has investigated the sedimentation of such material over a range of concentrations. The sedimentation constant at higher concentration differs little from that of undegraded hyaluronic acid complex; the variation with concentration is much less. The data available (S_{20} at zero concentration, taken as $2{\cdot}0 \times 10^{-13}$ and the variation of $1/S$ with concentration (Fessler & Ogston, 1951), taken as $0{\cdot}49 \times 10^{13}$, both from Hahn's data; and the intrinsic viscosity $(\eta_{rel.} - 1)\phi = 5{\cdot}9 \times 10^2$ from our results) enable estimates to be made of the molecular dimensions of the degraded material, using the procedure of Ogston & Stanier (1951*a*). The results are: $M = 107\,000$; effective volume = 5·5 ml./g.; axial ratio = 30. These results will apply to the average of the more highly polymerized part of a polydisperse mixture, since this part will make the greatest contribution to the viscosity and to the observed average sedimentation. They are consistent with the production, by hyaluronidase, of shorter chain units from a very large chain-polymer: the average unit has about 1/100 of the weight of the original polymer and would be expected to have a much lower degree of solvation (or entrainment of solvent) and a larger axial ratio.

Physical properties. Ogston & Stanier (1951*a*) assumed that hyaluronic acid complex constitutes 32 % of the weight of mucin, in calculating the intrinsic viscosity of the complex. The analyses reported here show that hyaluronic acid forms 45 % of mucin. It appears from Table 1 that the value of $\log\{(\eta_{rel.} - 1)/\phi\}$ at $\phi = 0$ should be between 3·9 and 4·0, instead of the value of 3·8 taken by Ogston & Stanier (1951*a*); this would have the effect of increasing the values of V' given in their Table 3 by a factor of 1·4. The value of the sedimentation constant, extrapolated to zero concentration is 14·7 instead of $13{\cdot}2 \times 10^{-13}$. This change, together with that of the values of V', will increase the values of M by a factor of 1·45, the values of a by 1·26 and of a^3 by 2·0. The calculated values of χ° and of Δ° are little affected. The new experimental value of $d(1/S)/dc$ is 3·3, instead of $3{\cdot}9 \times 10^{-3}$. None of these changes significantly affects the conclusions already drawn.

General conclusions. We briefly summarize our conclusions about the composition and properties of the hyaluronic acid of ox synovial fluid: (i) The hyaluronic acid occurs in the form of a complex containing 25 % of protein; the carbohydrate part of the complex consists mainly, perhaps entirely, of acetyl hexosamine and uronic acid (glucosamine and glucuronic acid, according to Meyer *et al.* (1939)). The molar ratio of hexuronic acid to hexosamine is 1·25–1·35. (ii) The complex is very easily degraded and only the mildest methods of separation (ultrafiltration and probably electrophoresis) preserve its native properties. (iii) The particle weight is about 10^7: the dynamic properties of solutions of the complex show that the particles are nearly spherical and are very highly hydrated. These properties are consistent with the view that the complex is, essentially, a flexible chain-polymer.

SUMMARY

1. Methods of separating hyaluronic acid from ox synovial fluid have been re-investigated, using the variation of viscosity with velocity gradient as a criterion of degradation. Precipitation as mucin

and precipitation by ethanol cause degradation, while ultrafiltration does not.

2. The course of purification by ultrafiltration has been followed. It has been confirmed that the protein content of the complex is reduced to a limiting value of 25 %. Electrophoresis is shown not to give products of lower protein content.

3. Analysis of the product of ultrafiltration shows that 93 % of its weight is accounted for by acetyl hexosamine, hexuronic acid, protein and ash. The protein content of the complex is found to be lower than was previously reported.

4. A revised value is given for the composition of mucin, prepared on a small scale.

5. Degradation by hyaluronidase has been shown to leave a considerable fraction of the material in a polymerized state (molecular weight of the order of 10^5).

6. The ultracentrifugal sedimentation of purified hyaluronic acid complex has been re-investigated.

7. Slight modifications of the values of physical quantities are given, based on the revised analytical values and sedimentation constant.

8. Our conclusions about the composition and molecular configuration of the hyaluronic acid complex are summarized.

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Note added in proof. Since this paper was sent in, some of our preparations have been examined by Dr R. Consden, who finds that small amounts of neutral sugars are consistently present.

REFERENCES

Blix, G. (1940). *Acta physiol. scand.* **1**, 29.
Cecil, R. (1951). *Biochem. J.* **49**, 183.
Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
Cecil, R. & Ogston, A. G. (1951). *J. Sci. Instrum.* **28**, 253.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Fessler, J. H. & Ogston, A. G. (1951). *Trans. Faraday Soc.* **47**, 667.
Hahn, L. (1947). *Biochem. Z.* **318**, 123.
Johnston, J. P., Ogston, A. G. & Stanier, J. E. (1951). *Analyst*, **76**, 88.
Karrer, P. & Schwarzenbach, G. (1934). *Helv. chim. Acta*, **17**, 58.
Katchalsky, A. (1951). *J. Polymer Sci.* **7**, 393.
Katchalsky, A. & Spitnik, P. (1947). *J. Polymer Sci.* **2**, 432.
Leyton, L. (1951). *Analyst*, **76**, 723.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Meyer, K., Smyth, E. M. & Dawson, M. H. (1939). *J. biol. Chem.* **128**, 319.
Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.
Ogston, A. G. & Stanier, J. E. (1951*a*). *Biochem. J.* **49**, 585.
Ogston, A. G. & Stanier, J. E. (1951*b*). *Biochem. J.* **49**, 591.
Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
Ropes, M. W., Robertson, W. v. B., Rossmeisl, E. C., Peabody, B. & Bauer, W. (1947). *Acta med. scand. Suppl.* **196**, 700.
Williams, R. J. & Kirby, H. (1948). *Science*, **107**, 481.

Separation of Adenosine Phosphates by Paper Chromatography and the Equilibrium Constant of the Myokinase System

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This paper describes a procedure for the quantitative separation by paper chromatography of adenosinetriphosphate (ATP), adenosinediphosphate (ADP), adenosinemonophosphate (AMP) and inorganic orthophosphate. The technique is a development of those of Hanes & Isherwood (1949) which are satisfactory for many purposes, but are inadequate for the complete separation of ADP and inorganic phosphate. The formic acid *iso*propyl ether solvent of Hanes & Isherwood separates satisfactorily the three adenosine phosphates from inorganic phosphate and the *iso*propanol ammonia solvent separates ATP from ADP, AMP and inorganic phosphate. The imperfect separation of ADP and inorganic phosphate is especially evident when the mixture of phosphates contains $KH_2{}^{32}PO_4$. The

latter can be detected by radioactivity when chemical methods are not sufficiently sensitive. Attempts to remove inorganic orthophosphate by precipitation as the magnesium ammonium salt lead to appreciable and variable losses of ATP and ADP by adsorption on to the precipitate.

Satisfactory separation of adenosine phosphates from other phosphates is achieved by a procedure which might be described as a 'two-solvents one-dimensional chromatography'. Two different solvents are used successively to develop the material in the same direction, the first ascending and the second descending on the reversed strip. This technique has the advantage over two-dimensional paper chromatography (of which it is a modification) that smaller pieces of paper are required and that a greater number can therefore be handled simultaneously. A further point is the greater compactness of the developed spots.

The method has been applied to the measurement of the purity of adenosine phosphate preparations and of the equilibrium in the myokinase system.

EXPERIMENTAL

Methods

ATP and ADP. Samples of ATP and ADP prepared from rabbit muscle (see LePage, 1949) and various commercial preparations were used. The barium compounds were dissolved in dilute HCl and barium was precipitated with the calculated quantity of Na_2SO_4. After removal of the precipitate by centrifugation the solutions were neutralized with NaOH. About 15–20% of the ATP was lost by adsorption on $BaSO_4$ (see Bailey, 1949). Calcium salts were treated similarly, sodium oxalate replacing sulphate.

Preparation of filter papers. Spots were more compact, and less 'trailing' occurred after treatment of the paper with ethylenediaminetetraacetic acid (which is expected to remove traces of metal impurities, see Walker & Warren, 1951). Whatman no. 1 papers, 45 × 19 cm., were immersed for 30 min., ten at a time, in a Perspex trough (22 × 60 × 5·5 cm.) in 2 l. 0·2% ethylenediaminetetraacetic acid, neutralized with NaOH to about pH 8·5. The trough was gently rocked at intervals. This was followed by six immersions, of 10 min. each, in 2 l. distilled water. The papers were then dried in a current of hot air.

An 'Agla' micrometer syringe (Burroughs Wellcome and Co.) was used to place 5 μl. ($\pm$0·005 μl.) quantities of the solution to be analysed, 4 cm. apart, on a line 8 cm. from one end of the paper. The spots were dried in a current of warm air from a hair dryer, care being taken to avoid a rise in temperature at the paper surface above 30°. Up to four additional 5 μl. quantities of the solution were added to the dry spot if necessary in order to accumulate 3–10 μg. P. The dry papers were bent at right angles along a line 6 cm. from the end, i.e. 2 cm. from the line of spots, and the other end of the paper was bent at right angles 2 cm. from the end. The 6 cm. margin was required for the second stage of the chromatography.

Preparation of biological material. Tissue extracts or suspensions were deproteinized at 0° by the addition of 0·1 vol. 30% (w/v) trichloroacetic acid and centrifuged at 2–4°. There were no changes in the ADP or ATP content of the supernatant solution after several weeks' storage at −14°. It was found to be unnecessary to neutralize these solutions for chromatography.

Chromatography. Chromatography was carried out in glass tanks of 40 cm. height and a base area of 30 × 22 cm., fitted with a plate-glass lid. (These are manufactured as jars for stationary accumulators and are available from Oldham and Son Ltd., Derbyshire House, St Chad's Street, London, W.C. 1.) Those used for descending chromatography were equipped with a glass-rod framework supporting two three-quarter cylindrical glass troughs, about 28 cm. long and capable of holding about 70–80 ml. solvent. A glass rod in the trough held the papers down in the solvent.

Two solvents recommended by Hanes & Isherwood (1949), one slightly modified by the addition of ethylenediaminetetraacetic acid, were used: (1) acid solvent consisting of 90 ml. *iso*propyl ether and 60 ml. 90% (v/v) formic acid; (2) alkaline solvent consisting of 240 ml. *n*-propanol, 120 ml. ammonia (sp.gr. 0·880) and 40 ml. 0·002 M-ethylenediaminetetraacetic acid.

The papers were fastened to the plate-glass lid with a strip of adhesive cellulose tape and were allowed to hang in the tank containing a 1–2 cm. layer of the acid solvent, the line of spots being within 2–3 cm. of the surface of the solvent. Up to eight papers could be placed in one jar simultaneously. The solvent was allowed to ascend for 3–4 hr. at 22° without preliminary equilibration, which proved unnecessary. After removal from the tank the papers were dried at room temperature in a fume cupboard by a current of air. The dried papers were serrated with scissors at the upper end and were placed in the reversed position into tanks fitted for descending chromatography. A shallow layer of the alkaline solvent was placed in the bottom of the tank and about 75 ml. into the trough. Four papers could be run simultaneously. The solvent was allowed to descend for 27–30 hr. at 28°. Preliminary equilibration proved again unnecessary. At the end of the run the papers were dried in a current of warm air.

Location of spots. Purines and pyrimidines were located by ultraviolet photography according to Markham & Smith (1949). Phosphate compounds were located by spraying with the molybdate reagents of Hanes & Isherwood (1949), followed by heating for 7 min. at 85° and exposure to H_2S.

Chemical analyses. For the quantitative determination of P an area of the filter paper containing the spots (4–10 sq.cm.) was cut out and wet ashed with 0·5 ml. of a mixture of H_2SO_4 (3 vol.) and $HClO_4$, sp.gr. 1·54 (2 vol.). P was determined in the ashed material by the method of Berenblum & Chain (1938) and colour intensities were measured in a Beckman spectrophotometer at 750 mμ. Some commercial samples of *iso*butanol required for the extraction were found to contain aldehydes and these were found to modify the colour from blue to greenish blue. *iso*Butanol was purified by shaking it with a solution of 2:4-dinitrophenylhydrazine in 10% (w/v) H_2SO_4 and distilling through a fractionating column. Paper blanks gave values of 0·01–0·04 μg. P/sq.cm. The lower limits of the visual detection of molybdate-sprayed spots were of the order of 0·07 μg. P/sq.cm. The ultraviolet technique for the detection of purines was more sensitive, the lower limit being 0·25 μg./sq.cm. adenylic acid, equivalent to about 0·02 μg. P/sq.cm.

To confirm the identity of adenosine phosphates determinations of ribose were made on the spots located in ultraviolet light. After elution of the cut-out paper with 4 ml. water for 1 hr. at 37° a 3 ml. sample was treated with orcinol

according to Meijbaum (1939). Paper treated with two solvents gave blanks equivalent to 0·3–0·6 μg. 'ribose'/sq.cm. This was higher than the blanks for untreated paper.

Adenine was not determined. The method of Cohn & Carter (1950) is not applicable to paper containing ethylenediaminetetraacetic acid. Paper blanks were carried out in all determinations on a relatively large piece usually about 15 sq.cm.

Radioactivity measurements. In some experiments solutions containing ^{32}P were analysed. The radioactivity was measured after wet ashing as described above and dilution to 10 ml. A β-counting tube, liquid type M6 of 20th Century Electronics, was used. Small quantities of orthophosphate contaminating the ADP spot can be detected by radioactivity when chemical methods fail.

Separation of phosphates

A diagrammatic representation of the chromatographic behaviour of various physiological phosphates is shown in Fig. 1. Strip *a* shows the separation of the three adenosine phosphates and orthophosphate by the new method. Strip *b* shows the behaviour of five other phosphates under the same conditions. It will be seen that the spots of 3-phosphoglyceric acid, glucose-1-phosphate and glucose-6-phosphate, inorganic pyrophosphate, fructose-1:6-diphosphate overlap partly with the adenosine phosphate spots and these substances may therefore interfere with the analysis of adenosine phosphates. The presence of these interfering substances can be detected by the ascending chromatogram in the acid solvent as shown on strip *c*. In the experiments reported in this paper the interfering substances were not present in significant quantities with the exception of pyrophosphate which overlaps with the ADP spot. The difficulty arising from the presence of pyrophosphate was overcome by separating pyrophosphate with the acid solvent, determining its quantity and making a correction in the analysis of the combined ADP plus pyrophosphate spot.

When both solvents were applied by the descending technique separation was incomplete and $KH_2{}^{32}PO_4$ was found to trail through the adenosine polyphosphate area.

Constancy and control of temperature is essential for satisfactory separation. At lower temperatures the development of the chromatogram is delayed.

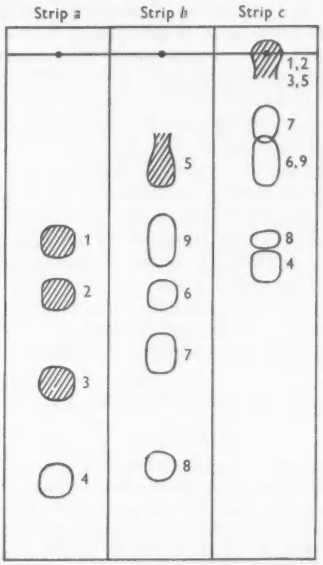

Fig. 1. Diagram of the position of phosphate compounds. Strips *a* and *b*, 3 hr. ascending in acid solvent at 22°, followed by 27 hr. descending in alkaline solvent at 28°. Strip *c*, 3 hr. in ascending acid solvent at 22°. (1) ATP; (2) ADP; (3) AMP; (4) inorganic orthophosphate; (5) unknown compound possibly inosine triphosphate (contains ribose and phosphate in the proportion of 1:3 and absorbs in the ultraviolet); (6) inorganic pyrophosphate; (7) glucose-1-phosphate and glucose-6-phosphate; (8) 3-phosphoglyceric acid; (9) fructose-1:6-diphosphate. Compounds which absorb in the ultraviolet are shaded.

Table 1. *Analysis of ATP and ADP preparations by 'two-solvent-one-dimensional' chromatography*

(Technique as described in text; 3–5 μl. of an 0·02 M solution of the Na salts were analysed; 'laboratory' preparations according to LePage, 1949.)

Preparation	Storage where known	Distribution of total P in the final solution (%)					
		ATP	ADP	AMP	Inorganic pyrophosphate	Orthophosphate	Other compounds
Barium ATP, commercial	—	42·5	39·9	0	7·3	0	10·3
ATP, free acid, commercial	5 months at 6°	68·8	21·8	3·7	0	0	5·7
Barium ATP, laboratory	8 months at −14°	86·0	11·0	0	0	3·0	0
Calcium ATP, commercial	2 weeks at 6°	32·6	24·3	9·6	7·4	16·2	9·9
ATP solution, commercial	—	30·1	24·6	0·5	0	28·2	16·6
Barium ATP, commercial	—	72·0	24·0	0	0	4·0	0
Barium ADP, laboratory	None	27·2	59·4	2·3	0	0	11·1
Barium ADP, labroatory	None	48·0	52·0	0	0	0	0
Barium ADP, laboratory (first Ba precipitation)	None	34·0	42·7	5·8	7·2	4·5	5·8

Concentrated extracts of biological materials sometimes contained sufficient Mg and Ca to give an additional inorganic phosphate spot located between ADP and AMP when run with the ammonia solvent. This has been prevented by the addition of 5 μl. 0·02 M-ethylenediaminetetraacetic acid to the spot before final drying.

Analysis of ATP and ADP preparations

None of the preparations tested, as will be seen from Table 1, was pure. All ATP preparations were contaminated with ADP and all ADP preparations with ATP. In the best preparations of ATP 11 % of the total P was ADP-P and 3 % inorganic phosphate. The samples of ADP were prepared by the action of adenosinetriphosphatase from pigeon-breast muscle or rat-leg muscle on ATP. The difficulty of complete hydrolysis of ATP has been noted by Bailey (1949).

Separation of radioactive inorganic phosphates

Table 2 shows the result of an experiment in which $KH_2{}^{32}PO_4$ was separated from the three adenosine phosphates. It will be seen that 99 % of the radioactivity was recovered in the orthophosphate spot, the traces found elsewhere being negligible. This degree of separation is satisfactory for experiments in which the incorporation of radioactive phosphate into adenosine phosphates is studied.

Equilibrium constant in the myokinase system

Rough data published by Kalckar (1943) give values of 0·37 and 0·25 for the equilibrium constant K of the myokinase system, at 30° and pH 7·5:

$$\frac{[ATP^{4-}]\,[AMP^{2-}]}{[ADP^{3-}]^2} = K.$$

The methods described in this paper permit a more accurate measurement of the equilibrium constant than was previously possible. The constant was determined because its value is of general interest in connexion with the free energy of hydrolysis of the two pyrophosphate bonds of ATP.

Table 2. *Separation of isotopic orthophosphate from a mixture of adenosine polyphosphates*

(The sample analysed contained 2–10 μg. P of each of the four phosphate compounds. The data refer to the radioactivity found on various areas of a chromatogram paper after running with acid and alkaline solvents consecutively.)

Area of paper	Radioactivity (counts/min.)	Percentage of total radioactivity on the paper
ATP	4	0·05
ADP	12	0·15
AMP	48	0·6
Orthophosphate	7705	99·0
Rest of paper	20	0·2

Myokinase was prepared according to Kalckar (1943), but pigeon-breast muscle was used instead of rabbit muscle.

Table 3. *Myokinase equilibrium at* 25° *and* pH 7·4

(For experimental conditions see text.)

Exp. no.	Period of incubation (min.)	Concentration ($\times 10^{-3}$ M) of ATP	ADP	AMP	$K = \frac{[ATP]\,[AMP]}{[ADP]^2}$
1	0	2·04	0·39	2·96	—
	20	1·14	2·28	2·04	0·446
	60	1·10	2·27	2·08	0·445
	90	1·14	2·26	1·92	0·430
2	0	2·30	0·71	2·06	—
	30	1·54	2·26	1·42	0·428
	90	1·54	2·23	1·28	0·398
3	0	2·68	0·84	4·50	—
	20	1·51	3·22	3·31	0·484
	30	1·48	3·16	3·31	0·490
	40	1·45	3·19	3·29	0·469
	50	1·51	3·32	3·38	0·458
	60	1·55	3·22	3·31	0·498
4	0	2·07	3·90	1·07	—
	50	2·48	3·12	1·57	0·400
	60	2·48	3·09	1·53	0·398
5	0	3·47	5·23	0	—
	20	4·48	3·28	1·06	0·441
	30	4·46	3·30	1·06	0·434
	40	4·50	3·27	1·06	0·446
	60	4·46	3·29	1·06	0·438
				Average	**0·444**

To avoid the presence of inorganic phosphate triethanolamine was used as a buffer. The 0·1 M buffer stock solution of pH 7·4 contained 10 ml. 1 M-triethanolamine and 6·8 ml. 1 N-HCl in 100 ml. A set of parallel tubes was incubated, each containing 0·15–0·6 mg. enzyme protein in a total volume of 2 ml. The final concentration of the sum of the adenosine phosphates was $5–10 \times 10^{-3}$ M, of $MgCl_2$ 0·01 M and of triethanolamine buffer 0·02 M. The exact quantities of the initial amounts of the adenosine phosphates are given in Table 3. At intervals samples were deproteinized with 0·2 ml. 30% (w/v) trichloroacetic acid and centrifuged at 2–4°. 15–25 μl. of the supernatants were used for chromatography. All analyses were carried out in duplicate which generally agreed within 3–4%, except in the AMP determination of Exp. 5 when the P values were very low. In this case the figure given is the average of eight determinations, the range being $0·94–1·18 \times 10^{-3}$ M.

It will be seen that, irrespective of the initial proportions of the adenosine polyphosphates, approximately the same value was obtained for the equilibrium constant, the average being 0·444. Thus the free energy liberated in the hydrolysis of the terminal pyrophosphate group under standard conditions is 480 cal. greater than that of the second pyrophosphate group.

DISCUSSION

Cohn & Carter (1950) separate adenosine phosphates by ion-exchange resins, which leads to large volumes of fluid which are inconvenient for the determination of phosphate and ribose. Alternatively, they use paper chromatography with solvents containing phosphate which make the simultaneous determination of adenosine phosphate and inorganic phosphate impossible. Two other methods were published after the completion of the present work. Bock & Alberty (1951) separate adenosine phosphates by a moving boundary method in the Tiselius apparatus, and Caldwell (1952) uses two-dimensional paper chromatography. The present method is simpler than these.

SUMMARY

1. A method for separating adenosinetriphosphate, adenosinediphosphate, adenosinemonophosphate and inorganic phosphate by paper chromatography is described. Two different solvents are used successively to develop the spots in the same direction.

2. The method has been applied to the analysis of adenosine phosphate preparations and to the determination of the equilibrium constant of the myokinase system. The value of K (as defined on p. 159) was found to be 0·444.

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REFERENCES

Bailey, K. (1949). *Biochem. J.* **45**, 479.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Bock, R. M. & Alberty, R. A. (1951). *J. biol. Chem.* **193**, 435.
Caldwell, P. C. (1952). *Biochem. J.* **50**, xxxv.
Cohn, W. E. & Carter, C. E. (1950). *J. Amer. chem. Soc.* **72**, 4273.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Kalckar, H. M. (1943). *J. biol. Chem.* **148**, 127.
LePage, G. A. (1949). *Biochemical Preparations*, vol. I. New York: John Wiley and Sons, Inc.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Meijbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Walker, D. G. & Warren, F. L. (1951). *Biochem. J.* **49**, xxi.